ELECTRON MICROSCOPIC ANALYSIS OF THE EFFECTS OF PSORALEN AND INTERFERON ON REPLICATIVE INTERMEDIATES FORMED DURING ENCEPHALOMYOCARDITIS VIRUS INFECTION OF MOUSE L CELLS

1987

O'CONNOR

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APPROVAL SHEET

Title of Thesis: Electron Microscopic Analysis of the Effects of Psoralen and Interferon on Replicative Intermediates Formed During Encephalomyocarditis Virus Infection of Mouse L Cells

Name of Candidate: Mary L. O'Connor
Master of Science Degree
August 24, 1987

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ABSTRACT

Title of Thesis: **Electron microscopic analysis of the effects of psoralen and interferon on replicative intermediates formed during encephalomyocarditis virus infection of mouse L cells**

Mary Leslie O'Connor, Master of Science, 1987

Thesis directed by: Margaret I. Johnston, Ph.D.

Department of Biochemistry

Demonstration of the existence of base-paired RNA in replication intermediates of murine encephalomyocaritis virus is important in understanding the mechanism of viral replication, interferon induction, and the role of double-stranded RNA-requiring enzymes in the mode of interferon action. The psoralen derivative 4'-aminomethyl-4,5',8-tri-methylpsoralen was used to specifically modify possible base-paired regions of the replicative intermediate formed during encephalomyocarditis virus infection. Psoralen modified replicative intermediates were shown to be formed in-vitro and in intact cells. Psoralen-modified replicative intermediates were isolated and examined in the electron microscope. Electron microscopic analysis suggested that encephalomyocarditis replicative intermediates contain significant stretches of base-pairs in intact cells. Further, replicative intermediates from interferon-treated cells were significantly shorter than those from control cells. This result suggests that the interferon-treatment led to viral RNA

degradation, possibly through production and action of 2',5'-oligoadenylate. This study provides direct evidence that replicative intermediates of encephalomyocarditis virus contain base-pairs in intact cells, and are degraded as a result of interferon treatment.

ELECTRON MICROSCOPIC ANALYSIS OF THE EFFECTS OF PSORALEN AND INTERFERON ON REPLICATIVE INTERMEDIATES FORMED DURING ENCEPHALOMYOCARDITIS VIRUS INFECTION OF MOUSE L CELLS

by

Mary Leslie O'Connor

Thesis submitted to the Faculty of the Department
of Biochemistry Graduate Program of the
Uniformed Services University of the Health Sciences
in partial fulfillment of the requirements for the degree of
Master of Sciences, 1987

DEDICATION

To my family

ACKNOWLEDGEMENTS

I would like to sincerely thank my advisor, Dr. Margaret I. Johnston, for her assistance in completing this research. Her constant demand for quality and excellence was an invaluable asset. I also thank Dr. Bill Ruyechan, for his instruction on the electron microscope, and Dr. Andy Holmes for his advice, constructive criticism, and useful suggestions received during the formation of my thesis. Additionally, my thanks are also extended to Karen Winestock for her technical assistance, friendship and unending humor, and to Donna Noldy and Carol Schwartz for their support and good spirit.

I would especially like to thank Dr. Lucy Chang for her friendship and guidance during my years as a graduate student. Her committment and standards as a scientist have become the guidelines for my future career.

I am grateful for the moral support I have received from my mother and brother, Mary and Richard, as well as my parents-in-law, Becky and Edward. Above all, I am gratefull for the love and the support I have received from my husband Ed. Without his assistance and constant encouragement this thesis would not have been possible.

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[1]**Abreviations:** AMT, 4'-aminomethyl trioxsalen or 4'-aminomethyl--4,5',8-trimethyl psoralen; EMCV, encephalomyocarditis virus; IFN, interferon; MEM, Minimum Essential Media; Pipes, 1.0 M Piperazine-N, N'-bis(2-ethanesulfonic acid); RI, replicative intermediate; RF, replicative form; RNase L, latent endoribonuclease; RSB, 10 mM NaCl, 10 mM Tris·HCl, pH 7.4, 1.5 mM $MgCl_2$; RSBV, RSB supplemented with 1 mM aurintricarboxylic acid, 10 mM vanadyl ribonucleoside complex, 0.1% SDS; 0.5% SDS Buffer, 10 mM NaCl, 100 mM Tris·HCl, pH 7.5, 1 mM EDTA, 0.5% SDS; Spinner Complete Media, Minimum Essential Media supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 0.25 ug/ml fungizone, 10% heat inactivated fetal calf serum; TCA/PP_i, 5% trichloroacetic Acid/0.2% pyrophosphate; TE, 0.02 M Tris·HCl, pH 8.5, 2 mM EDTA; TN, 150 mM NaCl, 10 mM Tris·HCl, pH 7.4; TNE, 0.15 M NaCl, 10 mM Tris·HCl, pH 8.3, 5 mM EDTA.

I. Introduction

I.A. Picornaviruses

The picornaviruses are ether-resistant, polyhedral, RNA-containing viruses of man and animals. The picornaviridae family consists of 5 genera: the enteroviruses, the cardioviruses, the human rhinoviruses, the equine rhinoviruses, and foot-and-mouth disease virus. These groups can be further subdivided on the basis of their stability under mildly acidic conditions. Those which retain their structural infectivity at pH 3 include the enteroviruses and cardioviruses, and those unstable at pH 3 include the rhinoviruses and foot-and-mouth disease virus. Those which are unstable at pH 3 are further distinguished by a higher buoyant density in CsCl (Levintow, 1974, Baltimore, 1969, Madshus et al., 1984). The distinctive properties of these subdivisions presumably reflect significant differences between the members of this family, not only at the level of nucleotide sequences within the RNA molecule, but also with respect to their interactions with other structures. No corresponding differences in their mode of replication is apparent (Baltimore, 1969).

Biochemical analysis of picornaviruses became possible with the development of practical methods for the propagation of poliovirus in cultured cells, the development of plaque assays, definition of the essential nutrients in cultured cells, and the purification, crystalization and physicochemical characterization of poliovirus (Levintow, 1974).

Information on viral replication of the most commonly studied picornavirus, human poliovirus, was reviewed by Darnell and Eagle (1960). During the mid 1960's three major discoveries pertaining

to the molecular biological aspects of the picornavirus systems were reported: (i) induction of virus-specific, RNA-dependent RNA polymerase (Baltimore & Franklin, 1963) (ii) identification of a base-paired, double-helical replicative form of the viral RNA (Montagnier & Sanders, 1963), and (iii) translation of a polycystronic gene product and its subsequent cleavage into functional polypeptide units (Summers & Maizel, 1968).

In general, the biochemical process of infection begins with attachment of the virus, a roughly spherical particle of about 28-30 nm in diameter. No evidence exists for the presence of lipids as an integral component of the virion or of carbohydrate other than the ribose moiety of the RNA. A value of 8×10^6 daltons best represents the molecular weight of the virion, with approximately 30% representing RNA. Viral RNA is linear, possessing a unique uncapped 5' terminus. A sequence of adenylic acid residues of variable lengths appear immediately adjacent to the 3'-terminal adenosine (Merregaert et al., 1978, Giron et al., 1976, Fraenkel-Conrat, 1979, Richards & Ehrenfeld, 1980, Burness et al., 1977). Measurement of the length of poliovirus RNA by electron microscopy yields a molecular weight of about 2.6×10^6 daltons, corresponding roughly to 7500-8000 nucleotides (Hruby & Roberts, 1978, Granboulan & Girard, 1969).

According to Lonberg-Holm & Korant (1972), viral interaction with specific receptors is temperature dependent. Further, different picornaviruses react with different receptors (Medrano & Green, 1973, Miller et al., 1974). Once the virion becomes firmly attached, irreversible alterations occur that place the virus beyond the reach of neutralizing antibodies (Levintow, 1974 and references cited therein).

Subsequently, capsid proteins, mainly VP4, are lost with corresponding alterations in antigenic structure. These membrane-associated changes constitute the first steps in uncoating and release of the viral genome into the cytoplasm (Howe et al., 1980 and references therein).

The synthesis of viral proteins and RNA take place in the cytoplasm, as first reported by Franklin and Rosner (1962). A multi-stranded species of RNA is formed during the replicating cycle of picornaviruses, including mengo virus, poliovirus, and encephalomyocarditis virus, (EMCV)[1]. This species of RNA, commonly refered to as replicative intermediate (RI), is heterogenous in size and sedimentation coefficient, unlike genomic length viral RNA, is partly resistant to RNase, and is preferentially labeled early in infection by brief pulses of radioactive precursors, implying that it is the site of nascent RNA synthesis (Baltimore & Girard, 1966, Franklin, 1966, McDonnell & Levintow, 1970, Bishop & Levintow, 1971, Bishop & Koch, 1969). In view of the characteristic physicochemical and biological properties of the extracted multi-stranded forms of RNA, it has been proposed that the complementary strands of RNA within the cell, if not hydrogen-bonded to each other, may be extensively aligned adjacent to one another without hydrogen bonding. Replicating picornavirus RNA may consist of a double-stranded core with several single-stranded tails of variable length (Thach & Thach, 1973, Thach et al., 1974). Preparations of RI are insoluble in 1-2 M salt, indicating that substantial single-stranded regions are present. Upon thermal denaturation of purified RI, a biphasic curve is observed. First, a gradual rise in optical density occurs at relatively low temperatures, representing denaturation of the single-stranded tails, due to the

presence of secondary structure. At higher temperatures the optical density increases sharply, representing denaturation of the base-paired portion(s) of the molecule (Levintow,1974). The products of denaturation include (i) a fraction which is indistinguishable from the viral genome and (ii) a heterogenous population of smaller material. Electron micrographs of the structures corresponding to this model have been obtained. The "cores" correspond to viral genome length virus and have an average of 3-5 tails per core (Richards et al., 1984).

A second double-standed species has also been identified in RNA purified from picornavirus-infected cells. Unlike the RI, this species is uniform in size with twice the molecular weight of viral genomic RNA. It is completely resistant to RNase and its synthesis peaks late in the replicating cycle. This stucture is refered to as the replicative form (RF).

The mechanism of replication appears to be largely semiconservative-negative, or, synthesis of a negative strand from a positive strand. Replication is initiated with GTP which forms the 5'-triphosphate terminus. Chain growth is in the 5' to 3' direction, leaving a triphosphate at the 5' terminus. Both genomic RNA and nascent strands contain a triphosphate at their respective 5' ends with the nascent strands hydrogen bonded to the viral template (Bishop & Levintow, 1971, Levintow, 1974), (Figure 1).

The presence of double-stranded RNA in infected cells during viral replication has been considered extensively in picornavirus, influenza virus, vesicular stomatitis virus, as well as in several bacteriophages. In all cases three different RNA's are observed, a fully double-stranded RNA composed of a plus strand and a minus strand,

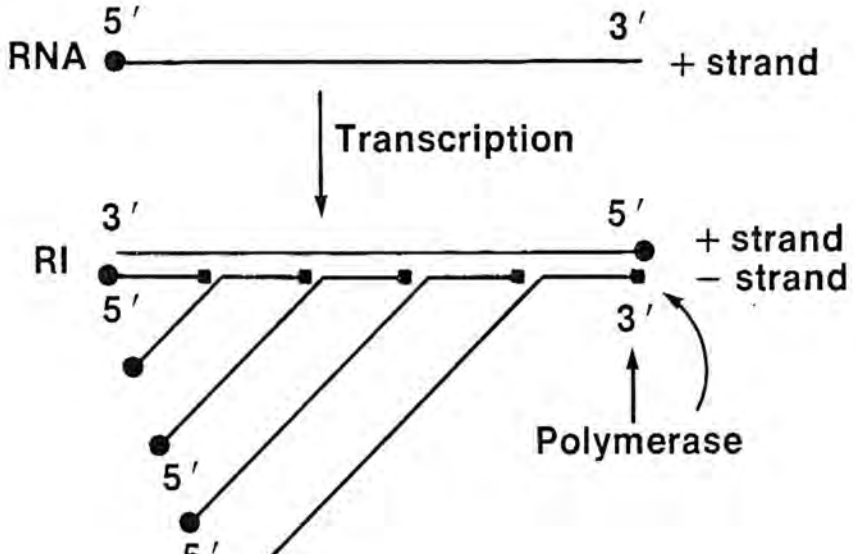


Figure 1. Semiconservative replication of picornavirus.

a RI RNA formed by a plus strand with nascent daughter strands, and molecules of single-stranded plus or minus strands (Johnston & Torrence, 1984). However, such structures have been observed in purified samples, and may not be present in the same form within the replicating complex inside the cell. Phenol deproteinization may be expected to collapse a replication complex and lead to artifactual formation of dsRNA.

I.B. Interferon Action

The presence of double-stranded regions during replication are important in that they may be the moiety responsible for induction of interferon (Torrence, 1985, Lengyel, 1982). Interferons are species-specific glycoproteins synthesized in a variety of cells by a variety of inducing agents including naturally-occuring and synthetic dsRNA. Interferons are powerful biological agents first studied as inhibitors of replication of almost all animal viruses (Isaacs & Lindenmann, 1975). Subsequently they were found to affect a variety of cellular functions, such as the ability to proliferate (Wells & Mallucci, 1985), to induce immunogenic responses, and to induce the synthesis of a variety of new proteins (Ratner et al., 1978).

Interferons are divided into three distinct groups: alpha, beta, and gamma. Alpha (α) interferon is mainly produced by myeloid cells, beta (ß) interferon by fibroblasts, and gamma (γ) interferon by T lymphocytes. Interferon α and ß can be induced by viruses, some bacteria and protozoa, natural double-standed RNA (dsRNA) such as Penicillium chrysogenum, as well as synthetic dsRNA such as

poly(I)·poly(C) (Ratner et al., 1978, Torrence, 1985, and references therein). Interferon γ is induced by tumor derived cells, mitogens, lectin stimulated T lymphocytes and natural killer cells.

In order for interferon to induce its effects it must first be secreted and bind to receptors on the cell membrane. Interferon binding to specific high affinity membrane bound receptors has been characterized by Zoon et al., (1982), and Anderson et al., (1982). Although all human interferons are similar in amino acid sequence and produce similar antiviral and antigrowth effects, they act through different receptors. Interferons α and β (type 1) bind to the same high affinity (10^{-10} M) species-specific receptors. These type I receptors are sparce (2-5000/cell) and are coded by a gene on chromosome 21. In contrast, interferon-γ acts through a more abundant type II interferon receptor (25-50,000/cell) that is not encoded by chromosome 21 (Weil et al., 1983, Aguet & Mogensen, 1983, Revel & Chebath, 1986). Binding of interferon (IFN) to its receptor causes a variety of cell regulatory effects, including induction of an antiviral state.

Elucidation of the antiviral action of IFN led to the discovery of two interferon-induced dsRNA-dependent enzymes, 2',5'-oligoadenylate synthetase and protein P_1 kinase. The protein P_1 kinase phosphorylates two substrates. One substrate is a protein of about 67,000 dalton which itself is the kinase, and the second substrate is initiation factor, eIF2. Phosphorylation of eIF2 sequesters the protein synthesis initiation factor in an inactive complex with eIF2B and results in an inhibition of translation of viral and host cellular

messenger RNA (Levin & London, 1978, Farrell et al., 1977, Clements & Williams, 1978, Kerr & Brown, 1978).

2',5'-oligoadenylate synthetase, of which several forms have been identified (Chebath et a., 1987, Ilson, et al., 1986, Benech et al., 1985, Nilsen et al., 1982a), is activated by dsRNA and polymerizes ATP into a family of 2',5'-linked oligoadenylates, $ppp^{5'}A(^{2'}p^{5'}A)_n$, (2-5A), where n is usually 1-3 (Baglioni et al., 1978, Slattery et al., 1979, Schmidt et al., 1978). 2-5A binds with high affinity and activates a latent endoribonuclease (RNase L), found in most vertebrate cells and tissue. In the presence of 2-5A, RNase L degrades RNA (Bayard et al., 1986, Rice et al., 1985, Meurs et al., 1985, Nilsen et al., 1981, reviewed, Johnston & Torrence, 1984, and references cited therein). The activity of the endonuclease appears to be controlled by the level of 2-5A in the cell (Dieffenback et al., 1985, Baglioni et al., 1984, Baglioni & Williams, 1985, Nilsen et al., 1982b). The 2-5A-mediated degradation of RNA may play a role in the IFN-induced antiviral action. In summary, degradation of RNA can be divided into two phases: an activation phase requiring the presence of ATP and dsRNA but not mRNA, and an endonucleolytic phase that does not require ATP or dsRNA. Nilsen & Baglioni (1979) reported that viral RNA is preferentially degraded in IFN treated extracts containing 2-5A synthetase and 2-5A dependent endonuclease (Figure 2).

One current model for 2-5A action proposes that localized synthesis of the 2',5'-oligoadenylate occurs where dsRNA is present. This leads to activation of the latent endoribonuclease and localized degradation of nascent viral strands (Baglioni et al., 1984, Nilsen &

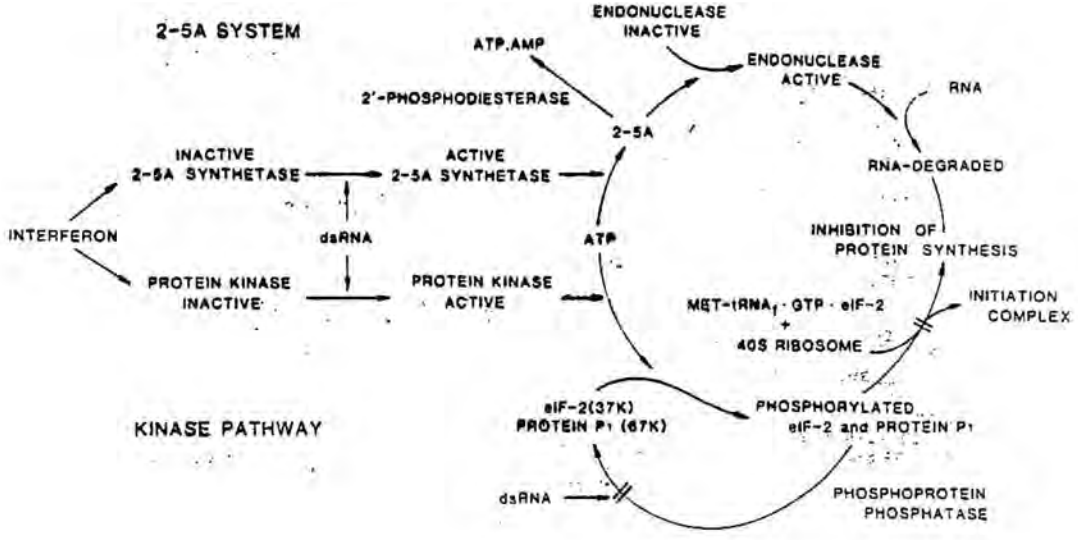


Figure 2. A schematic representation of the components and actions of the 2-5A system.

Baglioni, 1979, De Benedetti & Baglioni, 1984). Degradation products of the low molecular weight molecule, 2-5A, have also been observed. Inactivation of 2-5A appears to be caused by two other enzymes, a phosphatase and/or 2'-phosphodiesterase (Baglioni & Williams, 1985, Hearl & Johnston, 1987, Johnston & Hearl, 1987).

I.C. Psoralens

Formation of dsRNA regions during EMCV replication may be responsible for induction of interferon and activation of interferon-induced proteins. Isolation of the EMCV replicative intermediate allows analysis of the architecture of this viral stucture. However, formation of base-paired regions during purification of RNA may introduce artifacts. One approach would be to utilize a chemical derivative of psoralen to covalently modify based-paired RNA in the intact cell prior to cell disruption, and then to detect the presence or absence of such modifications.

Psoralens, or furocoumarins (Figure 3), (Hearst 1981), are naturally occuring planar tricyclic compounds formed by the fusion of a furan ring with coumarin. These compounds intercalate into helical nucleic acids and, upon irradiation with long wavelength UV light, react primarily with thymidine and uridine. Minor reactions with other bases have been reported. Psoralens and UV light are known to suppress cellular and viral DNA and/or RNA synthesis. As a chemical probe for studying molecules present in intact cells, psoralen posseses two important properties. Psoralens readily penetrate cells and they react with nucleic acids inside the cells. Thus, psoralens may covalently

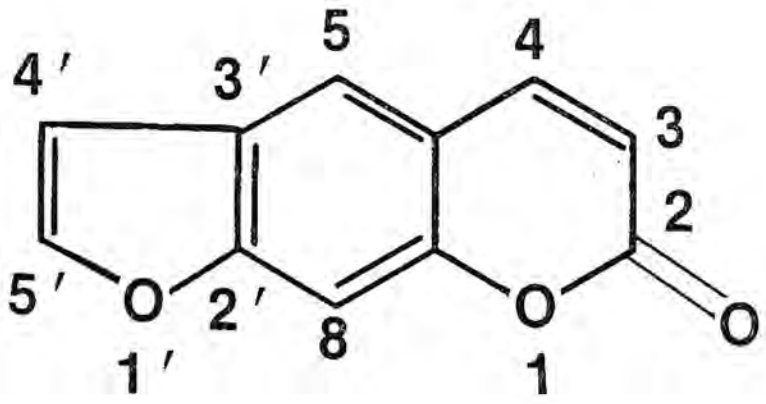
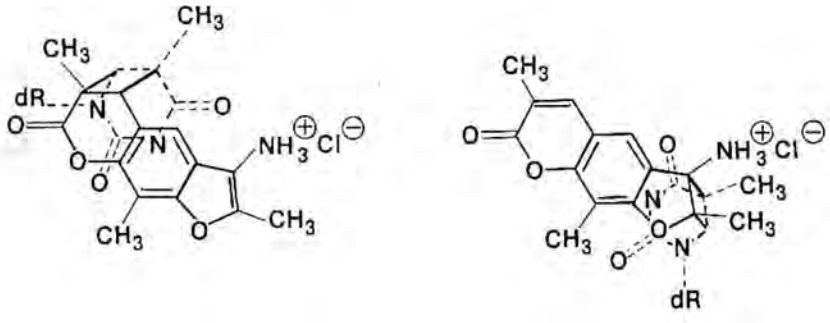


Figure 3. The structure of psoralen.

modify viral replicating complexes within the cell, and permit analysis of the RNA structures that exists during viral replication (reviewed, Cimino et al., 1985 and references therein).

The psoralen-nucleic acid reaction is a two-step photochemical process (Hanson et al., 1976). The initial step is intercalation of the psoralen molecule between base pairs in a nucleic acid helix. Upon UV-irradiation at 320-400 nm, the intercalated psoralen reacts with pyrimidine bases through cyclobutane additions forming psoralen-pyrimidine adducts. Stable, photoreversible (250 nm) covalent adducts can be formed by reaction of either end of the psoralen molecule (reviewed, Cimino et al., 1985). Because of this bifunctionality, two types of monoadducts may be formed. A furan-side monoadduct, M_{Fu}, is formed through reaction of the 4',5' double-bond of psoralen and the 5,6 double-bond of the pyrimidine base (Figure 4A). The pyrone-side monoadduct, M_{Py}, is formed through reaction of the 3,4 double bond of the psoralen and the 5,6 double bond of the pyrimidine base (Figure 4B), (Shi & Hearst, 1986, Bachellerie et al., 1981). When these reactions are properly sequenced, i.e. when cyclobutane addition occurs first at the furan-side, the monoadduct can absorb a second photon to form a diadduct if the psoralen is located at a cross-linkable site, such as occurs when another reactive pyrimidine base is located on the opposing strand of a double-helix (Figure 5), (Talib & Banerjee, 1982, Shi & Hearst, 1986). If the pyrone-adduct is formed first, it will not absorb other photons above 320 nm and will not form a diadduct.



A B

Figure 4. AMT monoadducts: (A) M_{fu} furan-side addition, (B) M_{Py} pyrone-side addition.

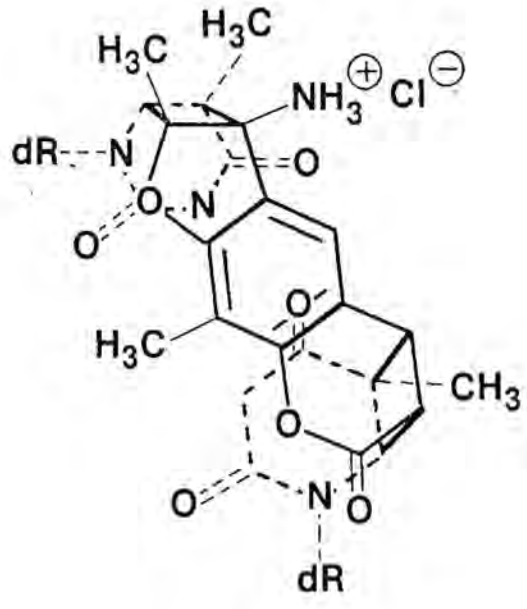


Figure 5. Structure of a diadduct formed by the reaction with thymidines.

The reaction of psoralens with RNA is similar to that of DNA, the major difference being the reactive base in RNA is uridine. The reaction of poly(U)·poly(A) and yeast RNA with the psoralen derivative HMT produces three major monoaddition products (Cimino et al., 1985 and references within). With each RNA, the major products were a pair of diasteriomers which were furan-side monoadducts. About 80% of the bound HMT reacted to form a monoadduct which could absorb another photon to form a cross-link. The third major product was a pyrone-side HMT-uridine monoadduct, which accounted for 20% of the total monoadduct formation in each reaction (reviewed, Cimino et al., 1985). The structure of the adducts formed between the pyrimidines and a variety of psoralens have been determined by mass spectrometry, ^{1}H-NMR, and X-ray crystallography for DNA. Complete characterization of uridine psoralen adducts or crosslinks remain to be determined (Shi & Hearst, 1986 and references therein).

The parent psoralen, (Figure 3) is not generally used due to its low solubility in biological solvents. A variety of compounds derived from the parent compound have been synthesized and are suitable for biological studies (Figure 6), (Hearst, 1981, Cimino et al., 1985). These compounds have been used previously to investigate the structural organization of chromatin (Ben-Hur & Elkin, 1973), ribosomes (Ceck & Karrer, 1980), 5S RNA (Thammana et al., 1979, Wollenzien et al., 1979), and viruses (Nilsen et al., 1981, Richards et al., 1984). One derivitized psoralen commonly employed is 4'-aminomethyl-4,5',8-trimethylpsoralen (AMT) (Figure 7), (Hearst, 1981). The advantages of using AMT are its greatly increased solubility in aqueous solvents and charged side chain. These factors

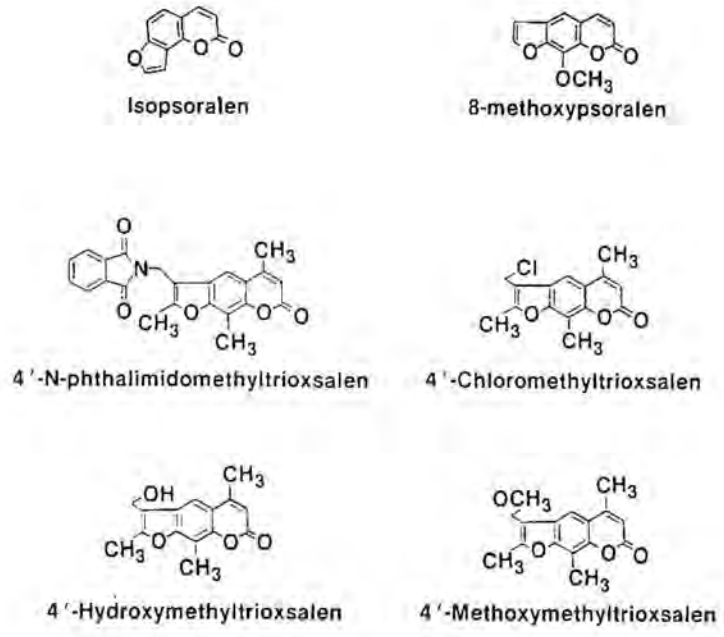


Figure 6. Structures of chemically synthesized derivatives of psoralen (Cimino et al., 1985).

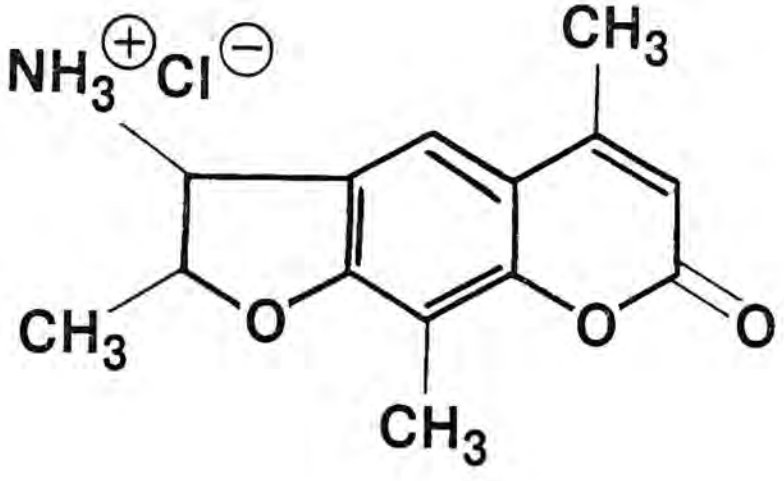


Figure 7. Structure of the psoralen derivative 4'-aminomethyl-4,5',8-trimethylpsoralen (AMT).

enhance binding of AMT to nucleic acids and facilitate intercalation of psoralen into a position required for photoreaction.

II. Research Goals

One goal of this research was to employ the psoralen derivative, AMT, to investigate the presence of base-paired regions that may occur in replicative intermediates formed during encephalomyocarditis virus replication. Studies would be performed to determine the concentration of AMT and time of irradiation that would result in psoralen addition to acid-precipitable nucleic acids in the cell. The effect of AMT on RNA synthesis in EMCV-infected cells would also be examined. Viral replicative intermediates would be purified and examined by electron microscopy. The presence or absence of psoralen modification may be determined visually or after reaction of the RNA with antibody directed against psoralen modified RNA. Psoralen modification, or lack thereof, would indicate the presence or absence of base-paired RNA in viral replicative intermediates.

It has been reported previously (Nilsen et al., 1981) that 2-5A synthetase requires a region of double-stranded RNA to become active and result in the specific RNase L-mediated degradation of viral RNA. A second goal was to isolate replicative intermediates from control and from interferon-pretreated cells to allow a direct comparison of the two replicative intermediate structures. If interferon treatment results in 2-5A production and action, the nascent strands of viral RI from interferon-pretreated cells would be expected to be shorter than those from control cells. Such a direct action of interferon on EMCV RNA has been suggested but not demonstrated directly.

III. Materials

III.A. Chemicals and Biochemicals

1. Actinomycin D: Actinomyces antibioticus, Grade III, from Sigma Chemical Company

2. Aurintricarboxylic Acid: Sigma Chemical Company

3. Aquassure: Pre-mixed scintillation solution for aqueous samples, New England Nuclear

4. Cytochrome C: VI, horse heart, Sigma Chemical Company

5. Dextran Sulfate: Sodium salt made from dextran with Mw 500,000, Pharmacia Fine Chemicals

6. Econoflour: Pre-mixed scintillation solution, New England Nuclear

7. 100% ethyl alcohol: dehydrated U.S.P., AAper Alcohol and Chemical Company

8. 95% ethyl alcohol: Todhunter International, Incorporated

9. Fetal Calf Serum screened for presence of bovine viral agents and mycoplasmas, M.A. Bioproducts

10. Formamide: Redistilled, nucleic acid grade, Bethesda Research Laboratories and M.A. Bioproducts

11. Glutamine 100x: 200 mM, 29.2 mg/ml, Gibco Laboratories

12. Glyoxal, trimeric dihydrate: Sigma Chemical Company

13. Hanks Balanced Salt Solution: Bioproducts

14. Minimum Essential Medium: Eagle modified for suspension cultures, Flow Laboratories

15. Nonidet P-40: Nonionic detergent, Sigma Chemical Company

16. Parlodion 3%-amyl acetate: EM Grade, Ernest F. Fullam Incorporated

17. Penicillin 10,000 u/ml, Streptomycin 10,000 u/ml, Fungizone 25u/ml, Advanced Biotechnologies Incorporated

18. Phenol: Redistilled, ultra pure, Bethesda Reseach Laboratories

19. Piperazine-N,N'-bis(2-ethanesulfonic acid) Sodium salt· 1/2 H_2O, Calbiochem/Behring Diagnostics

20. Pronase: Protease and nuclease free, 72,600 P.K.U./gm, Calbiochem/Behring Diagnostics

21. Recombinant Human Interferon-Alpha (A/D): Antiviral activity of $1.1x10^8$ U/ml, with a specific activity of $7.9x10^7$ U/mg, and a protein concentration of 1.4 mg/ml, Hoffmann LaRoche Incorporated

22. Trioxsalen, 4'-aminomethyl·HCl: 4'-(aminomethyl)-4,5',8-trimethylpsoralen, Calbiochem/Behring Diagnostics

23. Trioxsalen, 4'-aminomethyl·HCl: ^{3}H-AMT, 1 mCi/ml, specific activity: 10.5 Ci/mmol, HRI Associates

24. Trypsin-EDTA: Lyophylized Porcine parvovirus tested, 5.0 gm trypsin (1:250) and 2.0 gm EDTA/L in normal saline, 10x, Gibco Laboratories

25. Uranyl Acetate: Sigma Chemical Company

26. Uridine [5,6-^{3}H]: 35-50 Ci/mmol in aqueous solution, specific activity: 47.1 Ci/mmol, New England Nuclear

27. Uridine [^{14}C]: 50 uCi/ml in aqueous solution, specific activity: 453 mCi/ml, Amersham

28. Vanadyl Ribonucleoside Complex: 200 mM, Bethesda Research Laboratories

III.B. Other Materials

1. Agfa-Gevart print paper: Rapitone Type PF1-4, P1-4, Fuller D'Albert

2. Copper grids, 200 mesh: 3.0 mm diameter grids without center marking, Balzers

3. GF/C Filters: Glass fiber circles, 2.5 cm diameter, Whatman

4. Kodak EM Film: Fuller D'Albert

5. Mixed bed resin: Medium Porosity AG-501-18, 20-50 mesh, fully regenerated, BioRad

6. Pt/Pd: 80% Pt, 20% Pd, 0.008" diameter, Pelco

7. Qualitative filter paper: medium fast, 7.0 cm, Whatman

8. Radiometer Sensor: Model UVX-36-365 nm, UVP Incorporated

9. Sepharose CL-2B: Pharmacia Fine Chemicals

10. Sepharose 4B: Pharmacia Fine Chemicals

11. Tungsten V-filaments: 1 strand, Ted Pella Incorporated

12. UV source: Model B-100A Blak-Ray Lamp, UVP Incorporated

IV. Methods

IV.A. Cell culture

Mouse L cells were grown in 250 cm^2 flasks in Spinner Complete Media at 37°C, 5% CO_2, until they reached confluency. Cells were trypsinized and continued in spinner bottles seeded at $50x10^4$ cells/ml and incubated at 37°C. Cells were generally diluted to $20x10^4$ cells/ml every other day.

IV.B. Interferon-treated cultures

Cultures growing at 37°C in spinner flasks were diluted to $60x10^4$ cells/ml a day prior to infection. Recombinant Human Interferon Alpha-A/D (rHuIFN-αA/D), a hybrid interferon of HuIFN-A(α_2) and D(α_1) previously found to be active on mouse cells (Zoon & Wetzel 1984, Streuli et al., 1981, Weck et al., 1981), was added to a final concentration of 500 U/ml from a stock of $1.1x10^8$ U/ml. Interferon treatment was allowed to proceed for a minimun of 18 hours, after which cells were collected by centrifugation and infected at a multiplicity of infection of 20, for 3 hours. AMT modification and RI RNA isolation procedures were as described under sections IV.H. and IV.I., respectively.

IV.C. Measurement of RNA synthesis in virus-infected Murine L cells

Suspension cultures of mouse L cells were grown in Spinner Complete Media (MEM, 2 mM glutamine, 100 U/ml penicillin, 100 U/ml

streptomycin, 0.25 ug/ml fungizone. 10% heat inactivated fetal calf serum). Cells were concentrated to $5x10^6$ cells/ml and infected with purified encephalomyocarditis (EMC) virus at a multiplicity of infection of 10, 20 and 50, at 37°C. Viral infection was followed by measuring total ^{3}H-uridine incorporation in a 3 ml culture supplemented with actinomycin D (2 ug/ml) to inhibit host RNA synthesis and ^{3}H-uridine (20 uCi/ml) to label newly synthesized RNA. At defined time intervals, 100 or 150 ul aliquots, in duplicate or triplicate, were removed and placed in 1 ml of cold Hanks balanced salt solution on ice and vortexed. The cell suspension was centrifuged at 630 x g. The cell pellet was resuspended in 1 ml of water and vortexed vigorously to lyse the cells. Soluble RNA (20 ug/ml) was added as carrier and ^{3}H-uridine incorporation measured as described below. Cells remaining in the main culture were collected at the designated times post-infection by centrifugation at 800 rpm in an IEC centrifuge for 10 minutes and utilized in experiments described.

IV.D. Determination of acid precipitable radioactivity

Aliquots of water lysates were precipitated by adding 3 ml of 5% trichloroacetic acid/0.2% pyrophosphate (TCA/PP_i) and then filtered on Whatman GF/C filters (Lunquist & Maizel, 1978). The filters were washed twice with TCA/PP_i, dryed and added to 10 ml Econoflour and counted using a Model 3255 Packard Tri-Carb Liquid Scintillation Spectrophotometer.

IV.E. Effect of 4'-Aminomethyl-4,5'8,-trimethylpsoralen on RNA synthesis

Cells were grown and infected at 37°C, at a multiplicity of infection of 50. Cells were collected at 3 hours post-infection by centrifugation at 630 x g and suspended in TN buffer (10 mM Tris·HCl, pH 7.4, 150 mM NaCl) at $2.1x10^7$ cells/ml. The suspension was then subdivided into three groups (Richards et al., 1984).

Group I, minus AMT control: A 2 ml aliquot of the initial cell suspension with no added AMT was irradiated for 10 minutes under red light with long wavelength UV light (366 nm) using a Blak Ray Model B-100A lamp, at 15 mW/cm^2. Light intensity at the sample was measured with a model UVX-36-365 nm radiometer. Cells were maintained in the dark and then collected by centrifugation and resuspended in 2 ml of TN buffer (10 mM Tris·HCl, pH 7.4, 150 mM NaCl) supplemented with actinomycin D (2 ug/ml) and ^{3}H-uridine (20 uCi/ml). Duplicate aliquots of 150 ul were removed at time 0 and at 5 minute intervals for the next 30 minutes. Aliquots were placed in 1 ml of cold TN buffer and vortexed vigorously to lyse the cells. Soluble RNA was then added as carrier. The lysate was assayed for acid precipitable counts, as described in section IV.D.

Group II, minus UV light control: A second 2 ml aliquot of the initial cell suspension was centifuged at 630 x g and suspended in 2 ml of TN buffer supplemented with AMT (20 ug/ml), followed by 10 minutes under red light at 4°C. The cells were collected by centrifugation and resuspended in TN buffer supplemented with

actinomycin D (2 ug/ml) and ^{3}H-uridine (20 uCi/ml). As described above, duplicate aliquots were assayed at 5 minute intervals for ^{3}H-uridine incorporation by determining TCA precipitable counts.

Group III, sample: Cells were centrifuged at 630 x g and resuspended in 2 ml of TN buffer (10 mM Tris·HCl, pH 7.4, 150 mM NaCl) supplemented with AMT (20 ug/ml) and UV irradiated (366 nm) at 15 mW/cm^{2} for 10 minutes at 4^{o}C. Cells were collected by centrifugation, and suspended in 2 ml TN buffer containing actinomycin D (2 ug/ml) and ^{3}H-uridine (20 uCi/ml). Duplicate aliquots were assayed at 5 minute intervals for ^{3}H-uridine incorporation by determining TCA precipitable counts.

IV.F. Pulse labeling of viral RNA in Encephalomyocarditis virus (EMCV)-infected mouse cells

A suspension culture of mouse L cells growing at 37^{o}C in Spinner Complete Media was labeled overnight with ^{14}C-uridine at 10 nCi/ml to uniformly label cellular RNA. Cells were centrifuged and resuspended at 5x10^{6} cells/ml and then infected with EMCV at a multiplicity of infection of 20. Immediately after virus addition and at various times post-infection, 1 ml aliquots were removed from the main culture and incubated for 15 minutes with actinomycin D (7.5 ug/ml), followed by addition of ^{3}H-uridine at 20 uCi/ml and further incubation for 30 minutes (Nilsen et al., 1981). Aliquots (100 ul) were then removed from the 1 ml culture and placed in 1 ml cold Hanks balanced salt solution, vortexed and centrifuged at 960 x g for 10 minutes. The cell pellet was then resuspended in 1 ml of water and

vortexed vigorously to lyse the cells. Soluble RNA (20 ug) was added as carrier. The amount of newly synthesized RNA was determined by acid precipitation as described in section IV.D.

IV.G. Determination of the concentration of AMT required to block viral RNA synthesis

Suspension cultures of mouse L cells were labeled overnight with ^{14}C-uridine at 10 nCi/ml. Cells were concentrated at $5x10^6$ cells/ml, infected at a multiplicity of infection of 20, and incubated at 37°C. At 4 hours post-infection when viral RNA was about maximal, the cells were collected by centrifugation at 4°C and resuspended in TN buffer (10 mM Tris·HCl, pH 7.4, 150 mM NaCl). The cell suspension was aliquoted onto 24-well plates and treated with AMT at the following final concentrations: 0, 1, 2.5, 5, 10, 20, 50, 100, and 150 ug/ml. Each sample was individually UV irradiated (366 nm) at 15 mW/cm^2 for 15 minutes at 4°C. Control samples were also treated with AMT but were kept on ice under red light. Cells were collected by centrifugation and resuspended in TN buffer at $5x10^6$ cells/ml. The samples were then incubated in the presence of actinomycin D (7.5 ug/ml) for 15 minutes at 37°C, followed by addition of ^{3}H-uridine (20 uCi/ml) and further incubation at 37°C for 30 minutes. As described above ^{3}H-incorporation was assayed by TCA precipitation.

IV.H. Modification of RNA in intact cells with psoralen derivative

EMCV-infected or control cells were collected by centrifuga-

tion at 800 rpm for 10 minutes and resuspended in TN buffer (10 mM Tris·HCl, pH 7.4, 150 mM NaCl) at $2x10^7$ cells/ml. Cells were placed in a 25 cm^2 flask on ice. Under red light, AMT was added to 20 ug/ml from a stock of 2.5 mg/ml in 50% ethanol. Cells were UV-irradiated (366 nm) at 13-14 mW/cm^2 for 5 minutes (Richards et al., 1984). Identical additions of AMT followed by 5 minutes of irradiation were repeated 2 more times. Controls were treated with AMT as above but were kept on ice, under red light and not subjected to UV irradiation, for 5 minutes. RNA was isolated as described in section IV.I.

IV.I. Isolation of Encephalomyocarditis (EMC) virus replicative intermediate

Following AMT and irradiation treatments, cells were collected by centrifugation at 630 x g and washed twice with Hanks salt solution. Cell pellets were resuspended in RSBV buffer (10 mM NaCl, 10 mM Tris·HCl, pH 7.4, 1.5 mM $MgCl_2$, 1 mM aurintricarboxylic acid, 10 mM vanadyl ribonucleoside complex, 0.1% SDS) at $7.5x10^7$ cells/ml and vortexed. The cells were frozen at -70°C, thawed, and centrifuged at 11,500 rpm for 15 minutes. The supernatant was removed and kept at 4°C. The low speed pellet was resuspended in RSBV buffer, vortexed, and centrifuged at 11,500 rpm for 15 minutes. The low speed supernatants were pooled. Pronase was added to 500 ug/ml from a stock of 20 mg/ml. Cell sap (S10) was then incubated at 25°C for 2 hours with stirring. The mixture was extracted with an equal volume of TNE-saturated phenol warmed to room temperature. The aqueous layer was

removed and kept at 4°C. Interphase and phenol layers were extracted with an equal volume of TNE buffer (10 mM Tris·HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA) and aqueous phases were combined and re-extracted with TNE-saturated phenol. The aqueous phase was removed and adjusted to 0.2 M NaOAc. Nucleic acids were precipitated by addition of 2.5 volumes of cold 95% ethanol and incubation overnight at -20°C. The precipitate was collected by centrifugation at 11,500 rpm for 30 minutes, washed with cold 70% ethanol and then dissolved in TNE buffer. All single-stranded (ss) and partially double-stranded RNA were precipitated by addition of an equal volume of 4 M LiCl followed by incubation overnight at -20°C. The final pellet was dissolved in TN buffer (Richards & Ehrenfeld, 1980). In order to minimize RNA degradation all experimental procedures were conducted in siliconized and autoclaved glassware.

IV.J. In-vitro modification of viral RNA with psoralen derivative

Cells were collected by centrifugation at 800 x g for 5 minutes and resuspended in Spinner Complete Media supplemented with actinomycin D at 2 ug/ml, ^{3}H-uridine at 5 uCi/ml and purified EMC virus at a multiplicity of infection of 50. At three hours post-infection the cells were resuspended at 10^{8}cells/ml in RSB buffer (10 mM Tris·HCl, pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$). The cells were frozen at -70°C in a dry ice/ethanol bath, thawed in a room temperature water bath, and subjected to Dounce homogenization at 0°C. The lysate was centrifuged at 10,000 rpm for 10 minutes. The

supernatant was kept on ice and the pellet was resuspended in RSB buffer followed by centrifugation as above. The supernatants were pooled and DTT (dithiothreitol) was added to a final concentration of 1 mM. The S10 fraction was then placed in a 60 x 15 mm petri dish on ice and 4'-aminomethyltrioxsalen (AMT) was added to 200 ug/ml followed by 5 minutes of UV irradiation at 14.5 mW/cm^2. AMT and UV treatment was repeated two more times. Controls were treated as above but were not UV irradiated. The lysates were then supplemented with pronase at 500 ug/ml, aurintricarboxylic acid at a final concentration of 1 mM, and SDS at a final concentration of 0.1%. The cell mixture was stirred under red light for 2 hours. Samples were extracted with an equal volume of TNE-saturated phenol, vortexed and centrifuged. The phenol layers were extracted with TNE buffer, vortexed and centrifuged. The aqueous phases were combined and re-extracted with TNE-saturated phenol, vortexed and centrifuged. The aqueous phase was adjusted to 0.2 M NaOAc and 2.5 volumes of cold 95% ethanol were added. The nucleic acids were precipitated by overnight incubation at -20°C. The solution was centrifuged at 11,500 rpm for 30 minutes and the pellet was dissolved in TN buffer (10 mM Tris·HCl, pH 7.4, 150 mM NaCl). LiCl was added to a final concentration of 2 M and the mixture was incubated overnight at -20°C. Single-stranded RNA and partially double-stranded RI-RNA were obtained by centrifugation at 11,500 rpm for 30 minutes and applied to a Sepharose CL-2B column to isolate replicative intermediates (RI), as described in section IV.K.

IV.K. Purification of Encephalomyocarditis (EMC) virus replicative intermediate

To further separate replicative intermediates from other viral and cellular RNA, the LiCl pellet obtained as described above was subjected to one or two cycles of chromatography on Sepharose CL-2B. A column of 1.0 cm x 40.0 cm was eluted with 0.5% SDS buffer (10 mM NaCl, 10 mM Tris·HCl, pH 7.5, 1 mM EDTA, 0.5% SDS) at 6 ml/hr, with fractions collected every 10 minutes (Baltimore, 1967). The excluded peak fractions containing the partially double-stranded replicative intermediates (Spector & Baltimore, 1975) were pooled, precipitated with ethanol and employed in electron microscopic studies. Replicative intermediates from interferon and uninfected control cells were purified following the same procedures, except the LiCl pellet was subjected to only one cycle of chromatography on Sepharose CL-2B.

IV.L. Sample preparation for electron microscopy under denaturing conditions

RNA samples were denatured prior to spreading for electron microscopy. Denaturation was achieved by incubating 1 ug RNA (1 ul), 100 % formamide (7 ul), and 0.1 M $NaPO_4$ pH 7.4 (1 ul) at 70°C for 5 minutes. A 1 ul aliquot of glyoxal (6.9 M) was added and incubation was continued for 5 minutes at 70°C (Hsu et al., 1973, Richards et al, 1984). Samples were chilled quickly and incubated further at 4°C

overnight. RNA was spread from a hyperphase containing 70% formamide (v/v), 10 mM Pipes, pH 8.0, 1 mM EDTA, 50 ug cytochrome c, and 1 ug RNA in a total volume of 120 ul. Samples of 15 ul were layered onto a hypophase of 40% formamide (v/v), 10 mM Tris·HCl pH 7.4, 1 mM EDTA. The sample was spread as described in section IV.N.

IV.M. Sample preparation for electron microscopy under non-denaturing conditions

RNA samples, controls and psoralen-modified, were spread from a solution containing 1 ug/ml RNA, 30% formamide (v/v), 1 mM EDTA, 10 mM Tris·HCl, pH 8.5. These samples (15 ul) were layered on a hypophase of 10% formamide (v/v), 10 mM Tris·HCl, pH 7.4, 1 mM EDTA. The film was picked-up, stained, and shadow cast as described below (Meyer et al., 1978).

IV.N. Sample spreading for electron microscopy

Denatured and non-denatured samples (15 ul) were layered on to their respective hypophases as described above. RNA was picked up on 3% parlodion coated copper-200 mesh grids, stained with 0.5 uM uranyl acetate in 90% ethanol, rinsed, and dried in 2-methyl butane. ÒX174 was used as a length standard, and was added at 1 ug/ml prior to layering. Grids were rotary shadowed with Pt/Pd (80%/20%) at 8^{o}, and examined in a Zeiss electron microscope at 60kV. Photographs were taken on Kodak EM film. Prints were made on Agfa-Gevart Rapitone FP1-4 and P1-4.

IV.O. Formation of antigen-antibody complexes

Purified replicative intermediates from EMCV-infected L cells were obtained from pooled void volume fractions as described above. RI was incubated for 30 minutes at room temperature with purified rabbit IgG raised against psoralen-modified poly(A,U) (Hurt et al., 1987). The reaction mixture was chilled and treated with hot (90°C) formaldehyde for 15 minutes at a final concentration of .1%, and freshly diluted glutaraldehyde added to a final concentration of .5%. The mixture was passed through a 1.0 x 15.0 cm column of Sepharose 4B washed through with TNE buffer. The peak appearing just after the void volume contained the RNA-antibody complex (Stollar et al., 1986).

IV.P. Preparation of antigen-antibody complex for electron microscopy

Antibody-antigen mixtures (.035 ml) were prepared by the addition of 50 ul of 99% formamide, 10 ul of 1 M Tris·HCl, 0.1 M EDTA, 1 ug/ml of RNA sample and 50 ug of cytochrome c. A 15 ul aliquot of the above mixture was spread on distilled-deionized water. The film formed was picked up on parlodion coated grids, stained with uranyl acetate, and rotary shadowed with Pt/Pd as before (Nordheim et al., 1982).

V. Results and Discussion

V.A. Cell infection

Murine L cells grown in suspension were concentrated and infected at a multiplicity of infection of 10, 20 and 50. Spinner Complete Media was supplemented with actinomycin D to inhibit further cellular RNA synthesis, as well as ^{3}H-uridine to label any viral RNA synthesis. For nine hours post-infection, at hourly intervals, aliquots of the cell suspension were removed and assayed for ^{3}H incorporation as described. The results demonstrate that ^{3}H-uridine incorporation was similar for each multiplicity of infection (Figure 8). Due to the limited availability of the purified EMC virus ($2x10^{11}$ particles/ml), a multiplicity of infection of 20 was used in further experiments.

V.B. Time course of RNA synthesis in EMCV-infected cells

To determine the time course of RNA synthesis in EMCV-infected cells, murine L cells were pre-labeled with ^{14}C-uridine overnight in suspension cultures. Prior to infection, synthesis of cellular RNA was inhibited by the addition of actinomycin D as described in section IV.E. Viral RNA synthesis was monitored up to 7 hours post-infection by measuring ^{3}H-uridine incorporation. Cellular RNA was pre-labeled with ^{14}C-uridine, allowing the synthesis of viral RNA to be expressed as the ratio of ^{3}H to ^{14}C in precipitated RNA from cell lysates. Viral RNA synthesis increased rapidly between 3-4 hours post-infection and was maximal at 5-6 hours post-infection. After 6 hours the

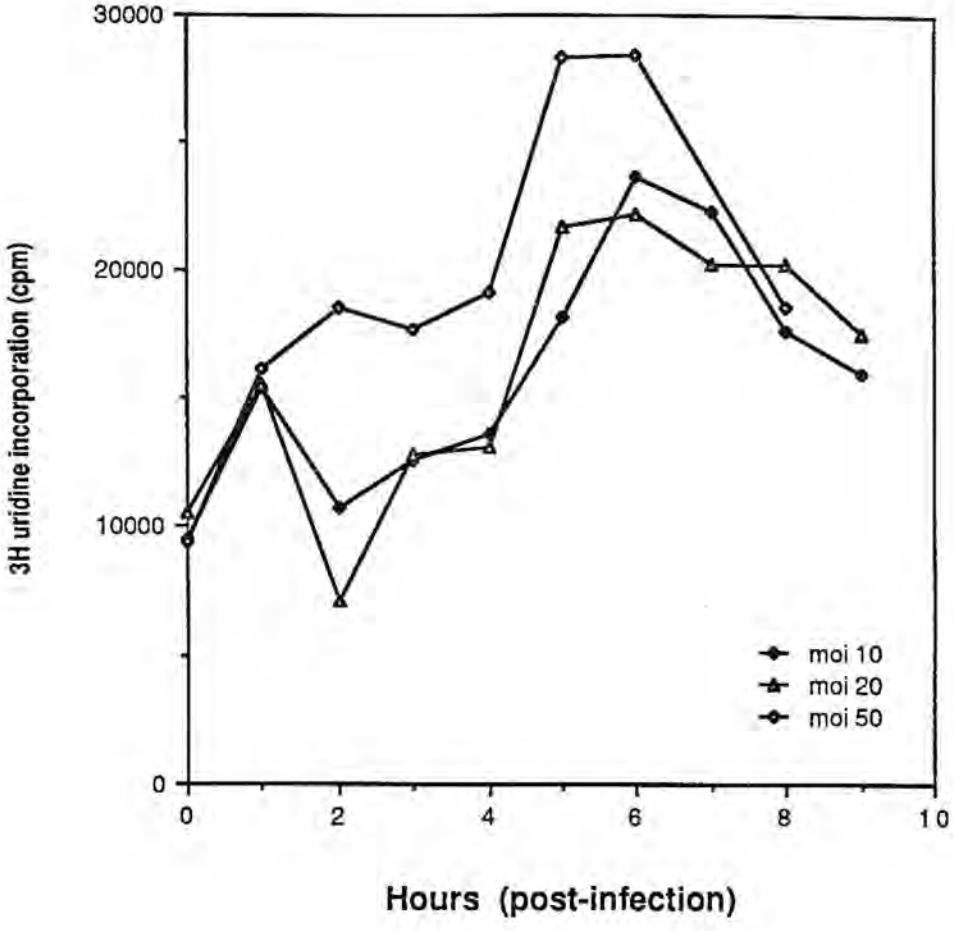


Figure 8. Time course of EMCV-infection in intact cells.

Murine L cells were concentrated at $5x10^6$ cells/ml and infected at three multiplicities of infection, 10 (◆), 20 (△) and 50 (◇). Cell media was supplemented with actinomycin D (2 ug/ml) and ^{3}H-uridine (20 uCi/ml). Samples (150 ul) were analyzed for TCA precipitable radioactivity as described in Methods.

precipitable radioactivity dropped dramatically (Figure 9). Cell viability was determined by dye exclusion at all time points. By 7 hours post-infection, 96% of the cells took up dye and were probably nonviable (Figure 10). Based on these results most infections were generally allowed to proceed up to 4 hours, at which time synthesis of replicative intermediates (RI) probably was occuring (Richards et al., 1984, Nilsen et al., 1981, Spector & Baltimore, 1975). Incorporation of ^{3}H-uridine observed at later times of infection probably represented synthesis of genomic RNA required for formation of daughter virions.

V.C. Inhibition of RNA synthesis by AMT as a function of time post-infection

Mouse L cells grown in spinner cultures were infected at a multiplicity of infection of 50. At three hours post-infection the cells were collected by centrifugation and resuspended in TN buffer. To ensure that AMT penetrated the cell membrane and modified RNA, cells were treated with AMT, with and without subsequent UV irradiation. The culture was divided into three groups: I, minus AMT control; II, minus UV control; III, plus AMT plus UV sample. Synthesis of cellular RNA was inhibited by actinomycin D and viral RNA synthesis was monitored by determining ^{3}H-uridine incorporation every 5 minutes for 30 minutes. Addition of AMT to control samples without subsequent UV irradiation resulted in a relatively linear synthesis of RNA up to at least 30 minutes post-labeling (Figure 11). RNA synthesis in cells that were not treated with AMT, but did receive UV-irradiation, decreased

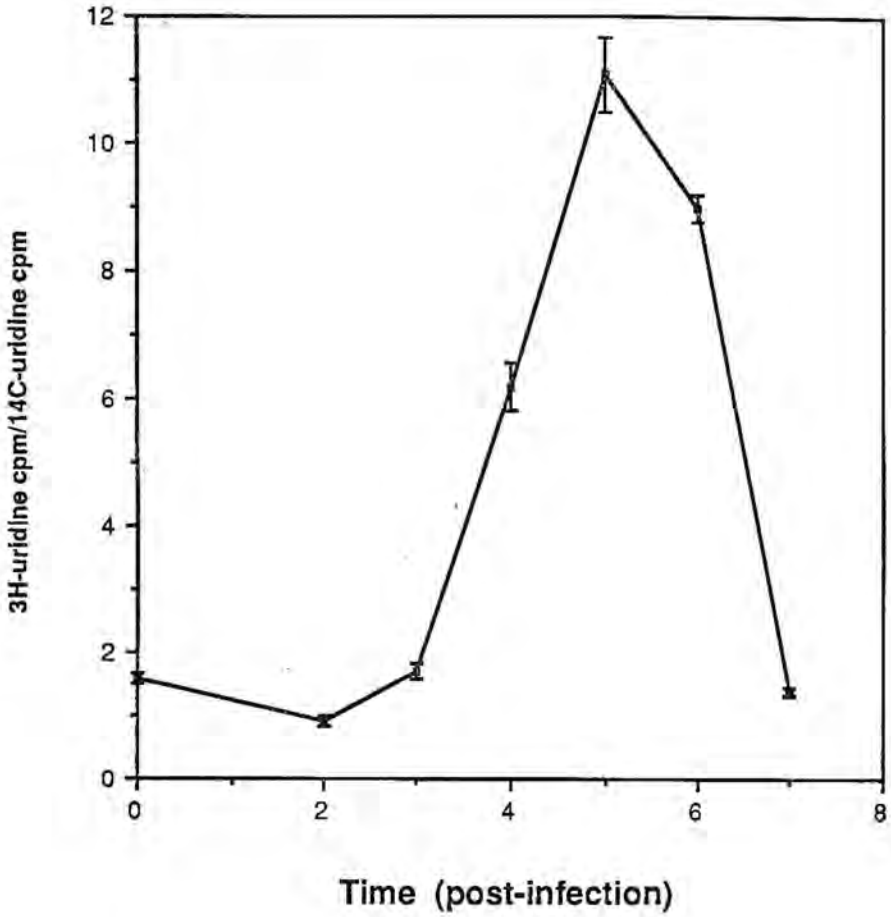


Figure 9. Pulse labeling of RNA during EMCV-infection.

Mouse L cells were prelabeled with ^{14}C-uridine and infected at a multiplicity of infection of 20. At each time point triplicate 1 ml subcultures were removed from the main culture and treated with actinomycin D (7.5 ug/ml) for 15 minutes at 37°C, followed by the addition of ^{3}H-uridine at 20 uCi/ml and further incubated for 30 minutes. Incorporation of radioactivity was assayed by TCA precipitation as described in Methods.

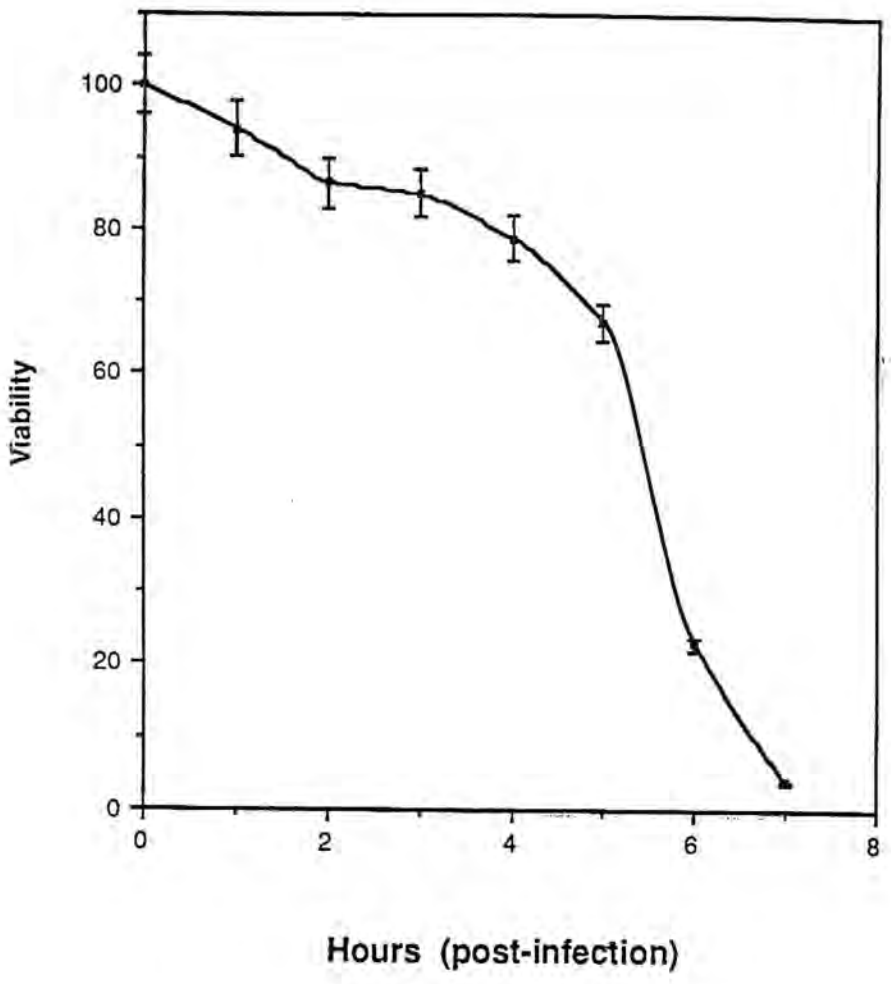


Figure 10. Cell viability during EMCV infection.

During an eight hour infection cycle 100 ul aliquots were removed from the main culture. Cells were stained with 0.2% trypan blue, any cells that took up dye were presumed to be dead.

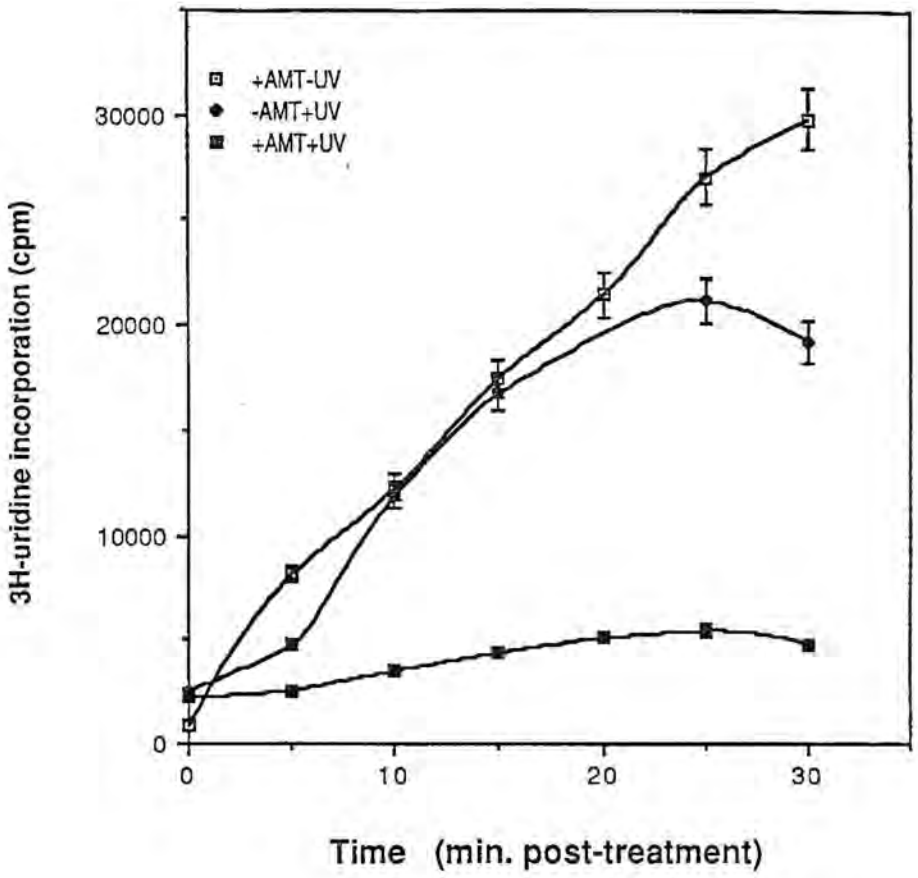


Figure 11. RNA synthesis in EMCV-infected cells.

Infected cells were collected at 4 hours post-infection, suspended in TN buffer at $2x10^7$ cells/ml and split into three equal subcultures. One group was supplemented with AMT to 20 ug/ml and kept in the dark ( ). A second group was irradiated with ultraviolet light (366 nm) at 4°C for 10 minutes ( ). The last group was supplemented with AMT to 20 ug/ml and irradiated with ultraviolet light at 4°C for 10 minutes ( ). All portions were collected and suspended in media containing actinomycin D (2 ug/ml) and 20 uCi ^{3}H uridine/ml. Aliquots were removed at indicated times and analyzed for trichloracetic acid precipitable radioactivity.

slightly by 20 minutes post-treatment. This may have resulted from uncharacterized UV-induced modification of the RNA. These two samples show relatively typical patterns of ^{3}H-uridine incorporation in virus-infected cells (Richards et al., 1984). In marked contrast, RNA synthesis in samples treated with AMT and UV light was strongly inhibited. These results indicate that AMT was penetrating the cells and was causing inhibition of RNA synthesis upon UV irradiation.

V.D. Inhibition of RNA synthesis as a function of AMT concentration

Cellular RNA was pre-labeled with ^{14}C-uridine overnight. Cells were concentrated in media and infected at a multiplicity of infection of 20. At 4 hours post-infection, cells were treated with AMT at final concentrations ranging from 0-150 ug/ml, followed by UV-irradiation. Synthesis of cellular RNA was inhibited by addition of actinomycin D, and viral RNA synthesis was followed by incorporation of ^{3}H-uridine into TCA precipitable counts. Control samples treated with AMT but not UV irradiated showed no significant difference in the amount of ^{3}H-uridine incorporated relative to that observed in the absence of AMT (Figure 12). Samples that were AMT-treated and UV-irradiated showed that 1 ug/ml AMT was sufficient to elicit a maximal decrease in further ^{3}H-uridine incorporation. Upon addition of 150 ug/ml of AMT there was no significant difference between irradiated and non-irradiated samples, demonstrating that high concentrations of AMT block RNA synthesis in the absence of

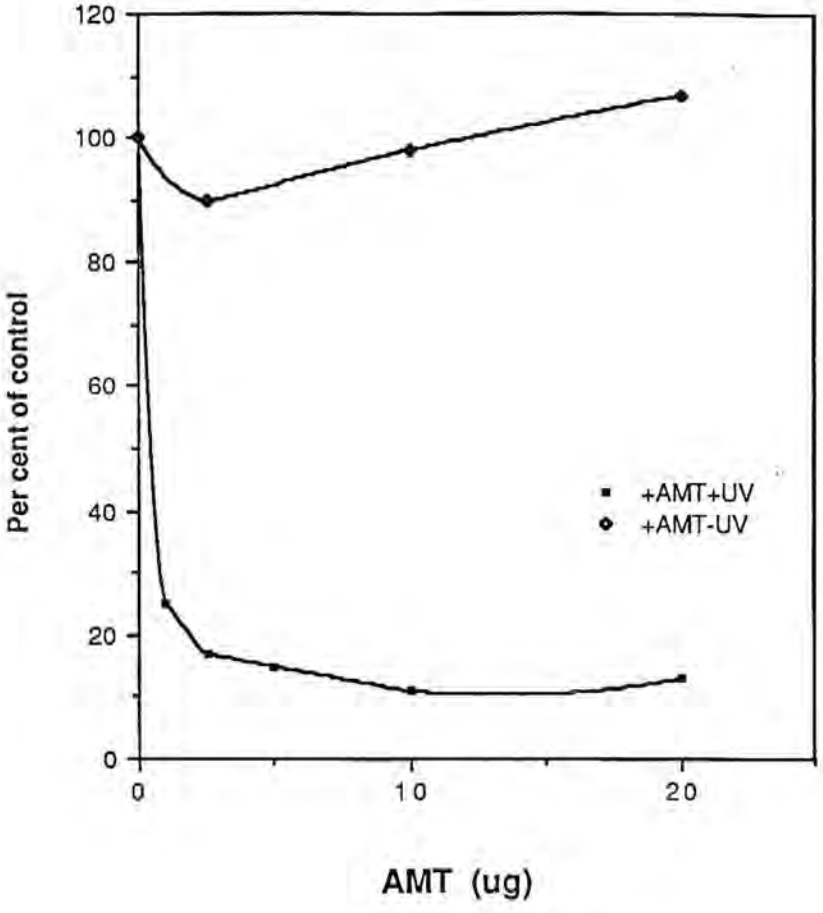


Figure 12. Effect of AMT on RNA synthesis in EMCV-infected cells.

Mouse L cells prelabeled with ^{14}C-uridine were infected with EMCV at a multiplicity of infection of 20, in the presence of actinomycin D (7.5 ug/ml). At four hours post-infection cells were collected by centrifugation and treated with varing concentrations of AMT, followed by UV-irradiation for 10 minutes ( ), or treated with AMT and not subjected to UV-irradiation ( ). Further RNA synthesis was labeled with ^{3}H-uridine for 30 minutes. Radioactive incorporation was assayed by TCA precipitation as described in Methods.

covalent modification (results not shown). In further pulse labeling experiments 5 ug/ml of AMT was employed. When isolation of the RI was performed, 20 ug/ml of AMT was used to maximize modification of the viral replicative intermediate.

V.E. Inhibition of viral RNA synthesis in AMT-treated cells as a function of UV-irradiation

Previous experiments demonstrated that AMT was penetrating cells and inhibiting RNA synthesis, and that 1 ug/ml of AMT was sufficient to cause such an effect. The length of irradiation required to photoactivate the psoralen to form covalent bonds with pyrimidines was then determined.

As in previous pulse labeling experiments cellular RNA was uniformly labeled with ^{14}C-uridine overnight. Cells were then concentrated and infected at a multiplicity of infection of 20. At 4 hours post-infection, cells were treated with 5 ug/ml of AMT and then irradiated for varied times. After AMT and UV-irradiation, samples were collected and resuspended in TN buffer containing actinomycin D and ^{3}H-uridine. RNA synthesis was followed by measuring ^{3}H-uridine incorporation and was expressed as the ratio of ^{3}H to ^{14}C (Figure 13). The results show that in control samples the presence of AMT alone without UV-irradiation (+AMT-UV), caused a very small decrease in viral RNA synthesis relative to other unirradiated samples. This sample and the zero time point for the +AMT +UV light were treated identically and gave identical results. Control samples subjected only to UV-irradiation without the addition of AMT (-AMT+UV) showed a very

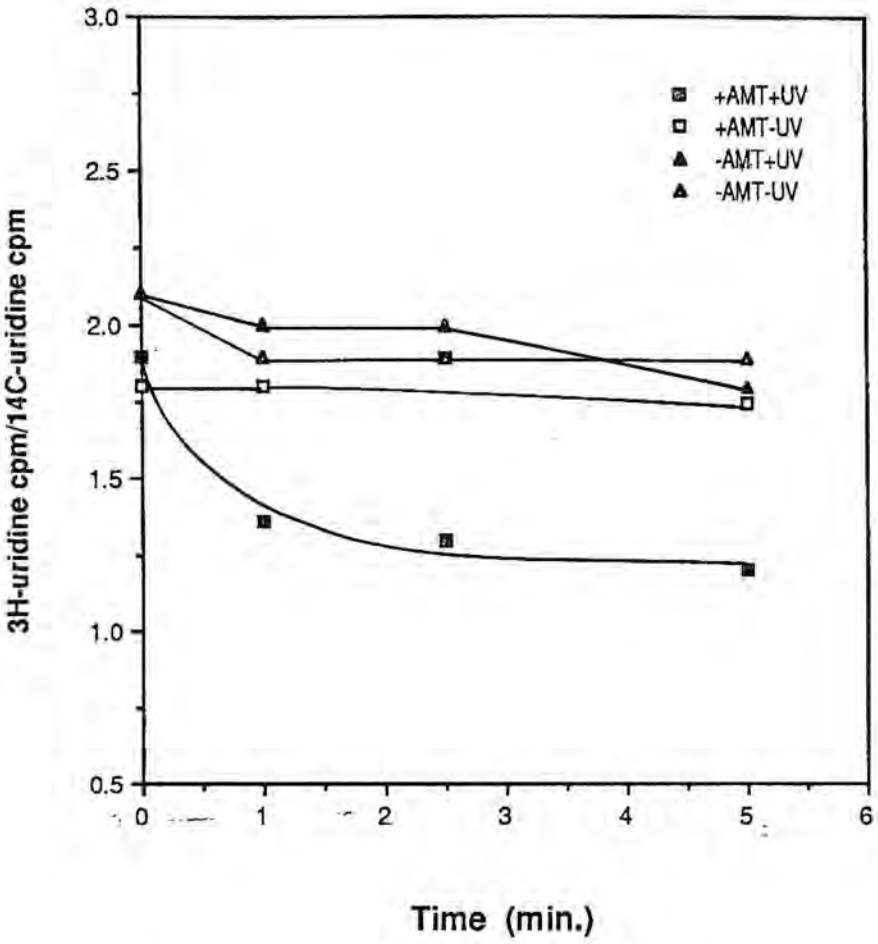


Figure 13. Effect of UV-irradiation on RNA synthesis in EMCV-infected cells treated and not treated with AMT.

Mouse L cells prelabeled with ^{14}C-uridine were infected at a multiplicity of infection of 20. At four hours post-infection the cells were subdivided into four groups and treated as follow: AMT (5 ug/ml) and no UV-irradiation (□), AMT followed by 10 minutes of UV-irradiation (■), no AMT and no UV-irradiation (△), and UV-irradiation treatment alone (▲). Cells were then suspended in Media containing actinomycin D (7.5 ug/ml) and incubated for 15 minutes. ^{3}H-uridine was added at 20 uCi/ml and further incubated for 30 minutes, labeled incorporation was assayed as decribed under Methods.

slight decrease in viral RNA synthesis with increasing exposure to UV light. These results agree with previously obtained data that suggests that UV alone will decrease viral RNA synthesis, as described above on the effects of AMT of RNA synthesis (Figure 11). Results obtained from samples treated with AMT and UV-irradiation, demonstrate that a 1 minute exposure to UV-irradiation was sufficient to depress RNA synthesis by 40% to as much as 73%, relative to non-UV-irradiated controls (Figure 13 and results not shown). This decrease in RNA synthesis was dramatic relative to all other treatments and controls. Because maximal inhibition of RNA synthesis occured by 5 minutes of UV-irradiation, it was decided that exposing cells to this length of UV-irradiation was probably sufficient to result in covalent modification of newly formed viral RNA.

V.F. Incorporation of ^{3}H-AMT into RNA in EMCV-infected cells

The results above suggest that AMT was probably modifing RNA in EMCV-infected cells. The nature of these RNA structures was not determined, but probably included newly synthesized viral RNA, since synthesis of RNA synthesized in the presence of actinomycin D was blocked by treatment with AMT and UV light (Figures 11 and 12). The following experiment was performed to definitively demonstrate that viral RI was modified with AMT. Cellular RNA was uniformly labeled with ^{14}C-uridine as described above. Cells were infected at a multiplicity of infection of 20 (as well as 50 in a separate experiment) in the presence of actinomycin D. ^{3}H-AMT was added to 5 ug/ml and a final specific activity of 50 uCi/ml. At 4 hours

post-infection the cells were UV-irradiated at 13 mW/cm^2 for 5 minutes on ice. The cells were collected and frozen at -70°C, thawed at room temperature and resuspended in RSBV buffer. Cytosolic fractions were obtained and RI was isolated as described in Methods.

Others demonstrated previously that viral RI elutes in the void volume of a Sepharose CL-2B column (Spector & Baltimore, 1975). Viral RNA eluting in the void volume, which constitute approximately 5-10% of total labeled RNA in infected cells (Nilsen & Baglioni, 1979), as well as other RNA eluting within the included volume of the column, was modified by ^{3}H-AMT (Figures 14 and 15). ^{14}C-uridine pre-labeled cellular RNA eluted as a single diffuse peak (results not shown). When AMT incorporation was expressed as the ratio of ^{3}H to ^{14}C, it appeared that under the conditions employed here, where cellular RNA synthesis was inhibited at least to some extent by actinomycin D, replicative intermediates were primarily modified (Figures 14 and 15). This result demonstrates that the viral RIs were modified by the psoralen compound. This finding was verified further by demonstrating that antibodies directed against psoralen-modified RNA bound RI isolated from EMCV-infected cells treated with AMT and UV light but not RI from similarly treated cells not UV-irradiated (results not shown, Hurt et al., 1987). It must also be noted that cellular rRNA is also modified by AMT, but possibly not to the same degree as the viral RIs.

The results described above provide valuable information on the time course and inhibition of the formation of EMCV replicative intermediates. This knowledge and techniques were utilized to isolate replicative intermediates formed in the cell during infection of control and interferon-pretreated murine L cells.

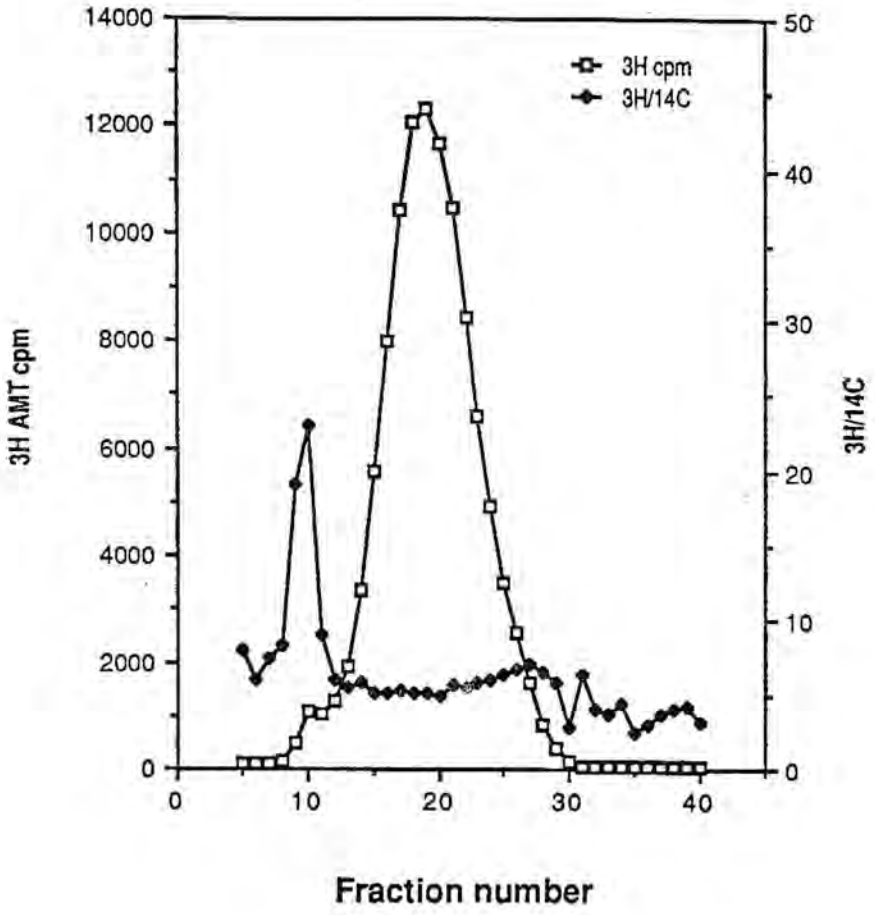


Figure 14. Sepharose CL-2B chromatography of ^{3}RNA from EMCV-infected cells treated with ^{3}H-AMT and UV light.

Murine L cells were prelabeled with ^{14}C-uridine and subsequently infected with EMCV for 4 hours at a multiplicity of infection of 20 in Media supplemented with ^{3}H-AMT ( ) and actinomycin D (7.5 ug/ml). At four hours post-infection cells were irradiated with UV light (14-15 mW/cm^{2}) for 10 minutes at 4^{o}C. Modified RI was purified and isolated as described in Methods.

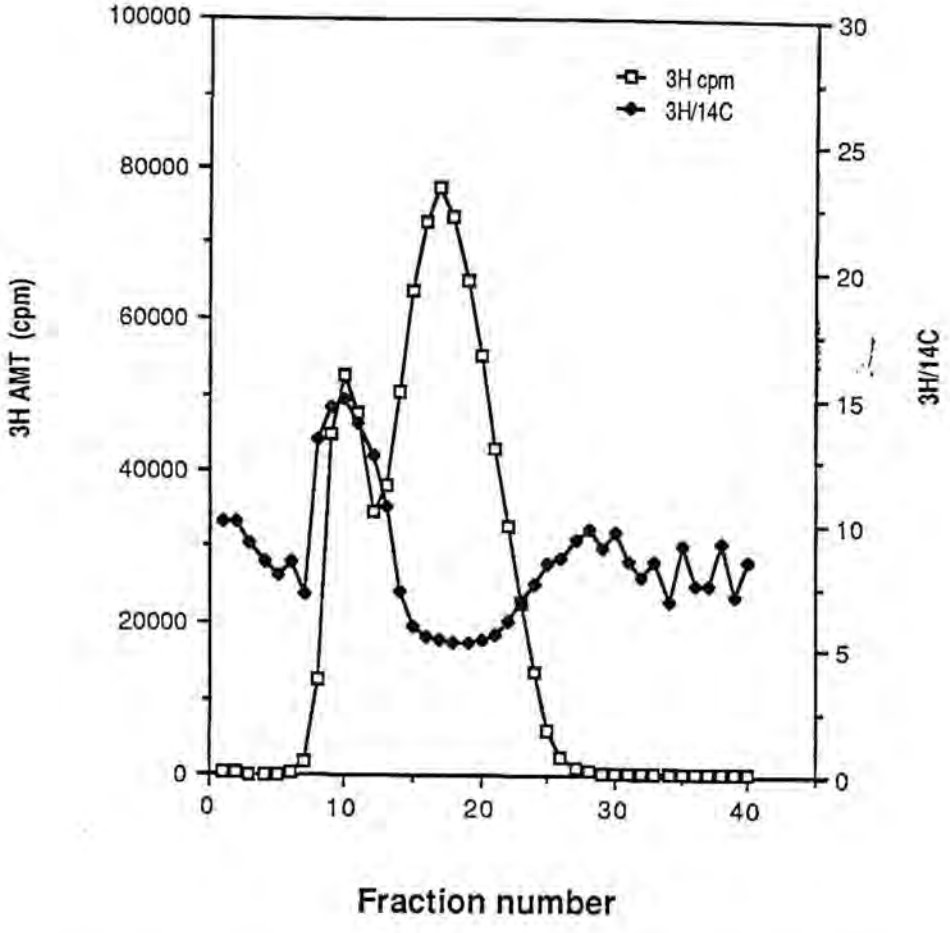


Figure 15. Sepharose CL-2B chromatography of RNA from EMCV-infected cells modified with ^{3}H-AMT and UV light.

Murine L cells were prelabeled as described under Figure 14 and subsequently infected under identical conditions, at a multiplicity of infection of 50. At four hours post infection cells were irradiated with UV light, RNA was isolated as described under Methods.

V.G. Isolation of Replicative Intermediates

Cytosol fractions of infected cells were phenol extracted to remove unreacted AMT as well as cellular proteins. Nucleic acids in these extracted fractions were ethanol precipitated, followed by fractional precipitation of single-stranded RNA and partially double-stranded RNA by addition of LiCl to a final concentration of 2 M. The precipitate containing single-stranded RNA and RI was then dissolved in TN buffer and subjected to chromatography on Sepharose CL-2B. As previously reported by Spector and Baltimore (1975), the RI's elute in the void volume (Figure 16). The void volume fractions were pooled and subjected to ethanol precipitation. To ensure the purity of the RI, this precipitate was subjected to a second cycle of chromatography on Sepharose CL-2B. One major peak was observed (Figure 17). Void volume fractions of this second cycle were pooled and subjected to ethanol precipitation. RNA was pelleted, dried, dissolved in TN buffer and utilized in EM studies.

Purification of RNA from interferon treated, EMCV-infected cells was achieved as described above with minor modifications. The RI peak seen in the first cycle of chromatography of control samples was not a well-defined peak but rather a shoulder on the major rRNA peak (Figure 18). Fractions 8-12 were pooled and subjected to ethanol precipitation. A second cycle of chromatography was not attempted due to the low yield of RI-RNA.

RNA from control uninfected cells was also subjected to the same isolation and purification procedures. No detectable RNA was observed in the void volume (Figure 19). This provided further evidence that the RNA isolated in the void volume for either control or

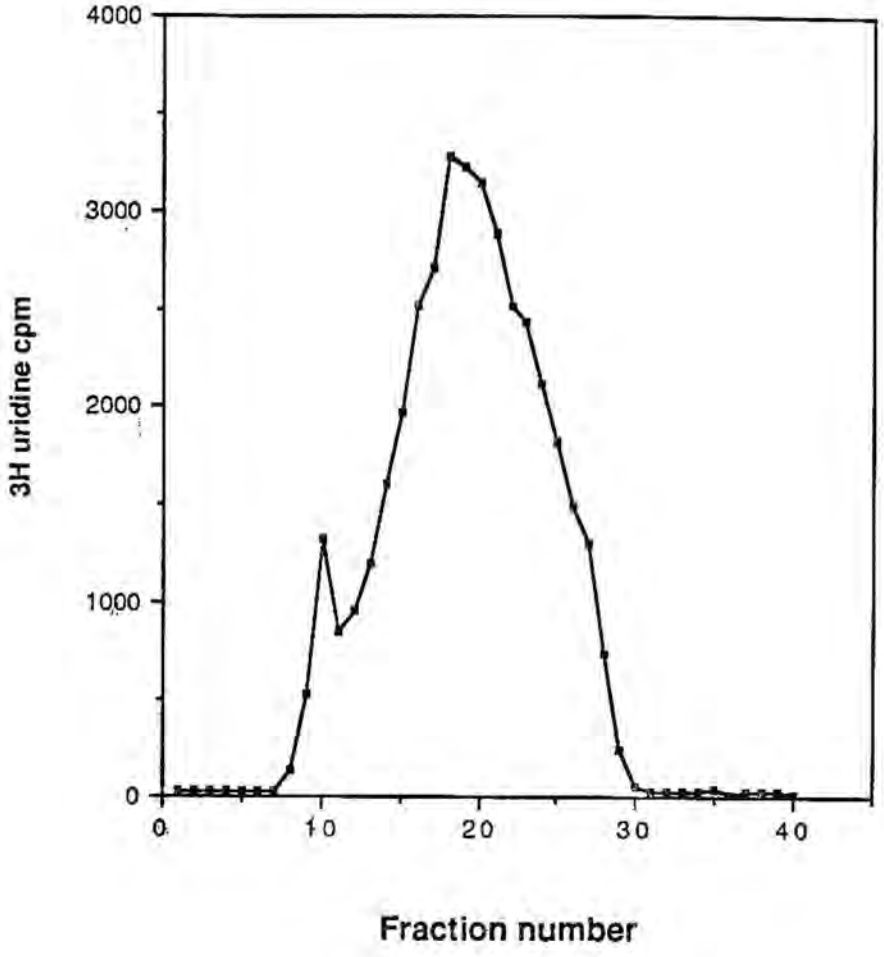


Figure 16. Sepharose CL-2B chromatography of RNA purified from EMCV-infected cells, treated with AMT, without UV-irradiation.

Mouse L cells were infected at a multiplicity of infection of 20, in Spinner Complete Media supplemented with actinomycin D 7.5 ug/ml) and ^{3}H-uridine (20 mCi/ml). At 4 hours post-infection cells were collected by centrifugation and treated with AMT followed by incubating at 4^{o}C for 5 minutes. RI eluting in the void volume, fractions 8-11, was purified and isolated as described under Methods.

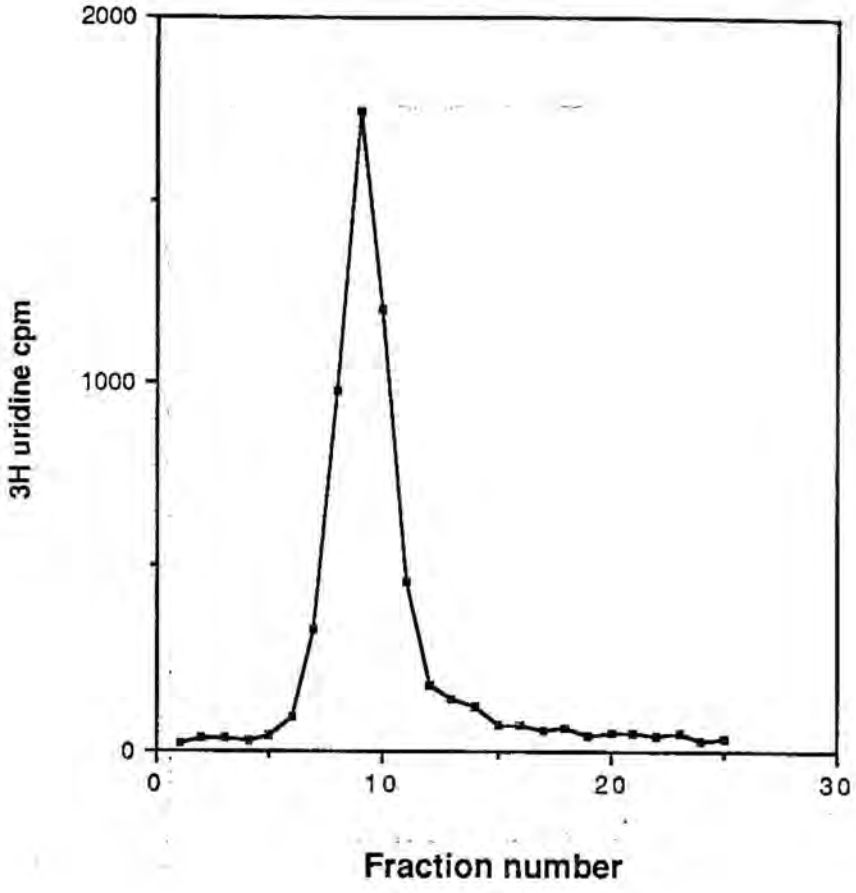


Figure 17. Sepharose CL-2B chromatography of purified EMCV replicative intermediate.

RNA purified from pooled fractions 8-11 (Figure 16), was subjected to a second cycle of chromatography on Sepharose CL-2B. Fractions 7-11 were pooled and used or electron microscopic studies as described in Methods.

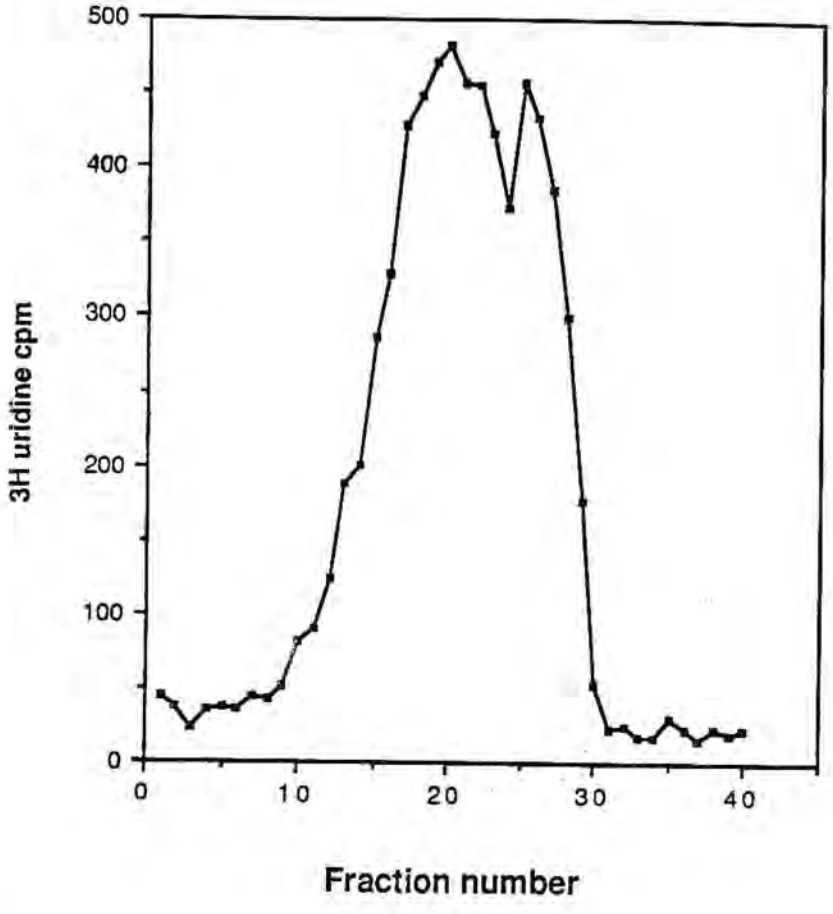


Figure 18. Sepharose CL-2B chromatography of RNA from interferon treated EMCV infected mouse L cells, treated with AMT, and UV light.

Cells were treated with 500 u/ml of rHuIFN- A/D for 18 hours prior infection. Cells were collected by centrifugation and infected at a multiplicity of infection of 20. At 4 hours post-infection the cells were collected and treated with AMT (20 ug/ml) followed by a 4°C incubation under red light. Fractions 8-13 were pooled and subjected to ethanol precipitation. Isolation and purification of the RI was as described in Methods.

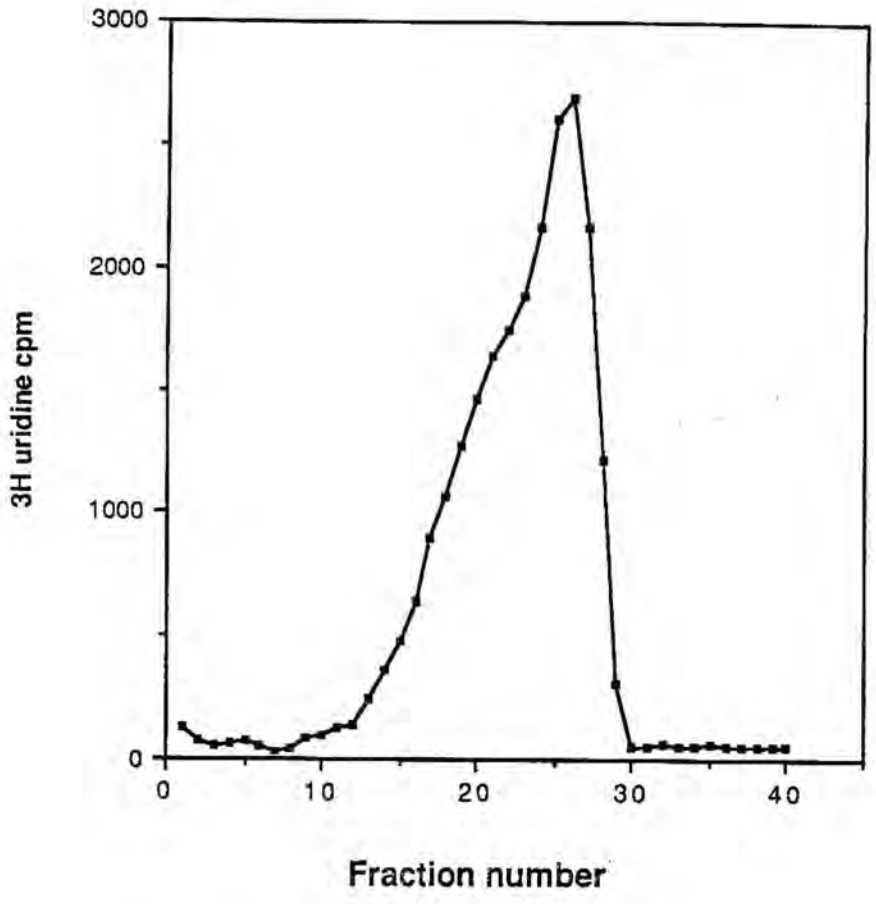


Figure 19. Sepharose CL-2B chromatography of RNA from mock-infected mouse L cells.

Mouse L cells were treated with ^{4}H-uridine (20uCi/ml) and actinomycin D (7.5 ug/ml) in Spinner Complete Media. At four hours post-treatment the cells were collected by centrifugation. RNA was isolated as described under Methods.

interferon-treated cells was EMC viral RI. Finally, elution profiles of RNA extracted from cells subjected to UV-irradiation (Figures 20 and 21) did not differ significantly from RNA extracted from non-irradiated cells (Figures 16 and 17).

V.H. EMCV replicative intermediates isolated from cells treated with AMT alone

When spread under denaturing conditions (Figure 22A,B), replicative intermediates obtained from EMCV-infected L cells treated with AMT alone, showed a range of sizes from .35 to 1.7 ΦX174 units with a mean size of 1.21 ± .36 ΦX174 units (Figure 23). Harsh denaturing conditions would be expected to diminish base pairing, and all single-stranded RNA would not appear to contain loops or hairpins. The smaller pieces (.35 to .70 ΦX174 units) may be incomplete daughter strands, whereas the longer pieces (.82 to 1.24 ΦX174 units) may be daughter strands near completion, and the longest strands are probably full genomic length EMCV RNA (1.41 to 1.64 ΦX174 units). Mature poliovirus RNA is approximately 7000-8000 bases in length (Hruby & Roberts, 1978). Using the internal standard ΦX174, the predicted length of mature EMCV RNA determined here is approximately 7560-8640 bases. As with previously reported data, measurements made were not corrected for a 26% decrease in length under denaturing conditions.

Replicative intermediates obtained after <u>in-vitro</u> treatment with AMT without UV-irradiation, and spread under denaturing conditions (Figure 24A,B) appeared very similar to the RIs obtained from intact cells sujected to a similar treatment (Figure 22). As

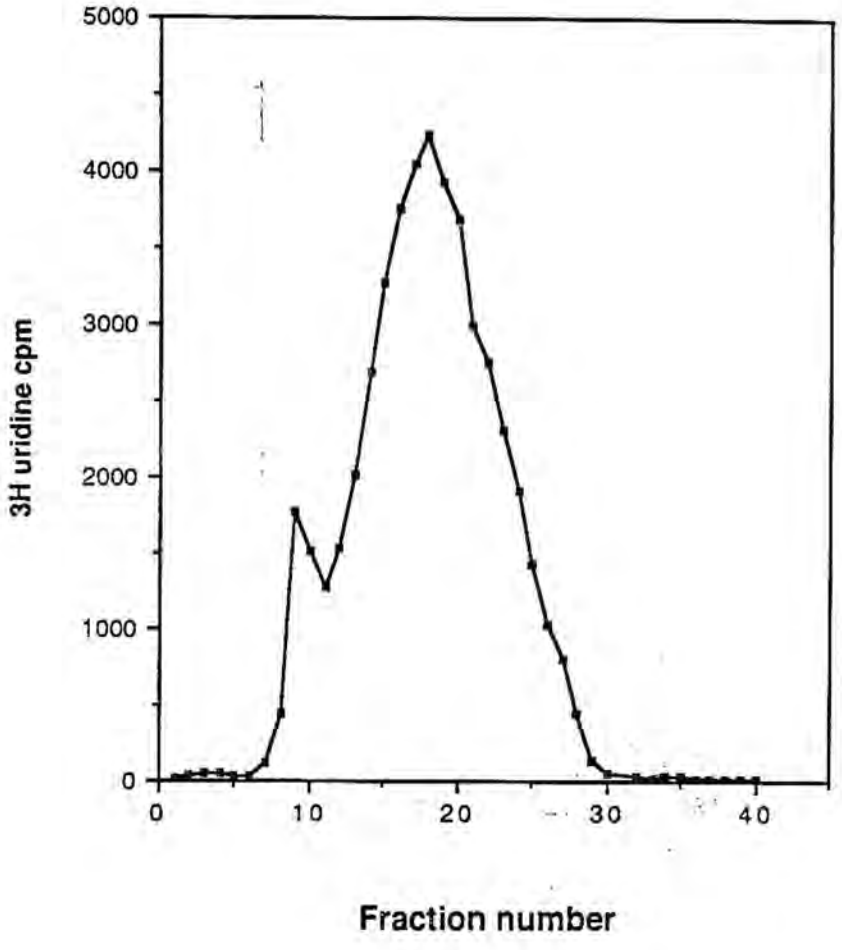


Figure 20. Sepharose CL-2B chromatography of RNA from EMCV-infected cells, treated with AMT and UV-irradiation.

Mouse L cells were infected at a multiplicity of infection of 20, in Spinner Complete Media supplemented with actinomycin D (7.5 ug/ml) and ^{3}H-uridine (20 uCi/ml). At 4 hours post-infection cells were collected by centrifugation and treated with AMT followed by UV-irradiation (13-14 mW/cm^2) at 4^{o}C for 5 minutes. RI was purified and isolated as described under Methods. Fractions 8-11 were pooled and sujected to ethanol precipitation.

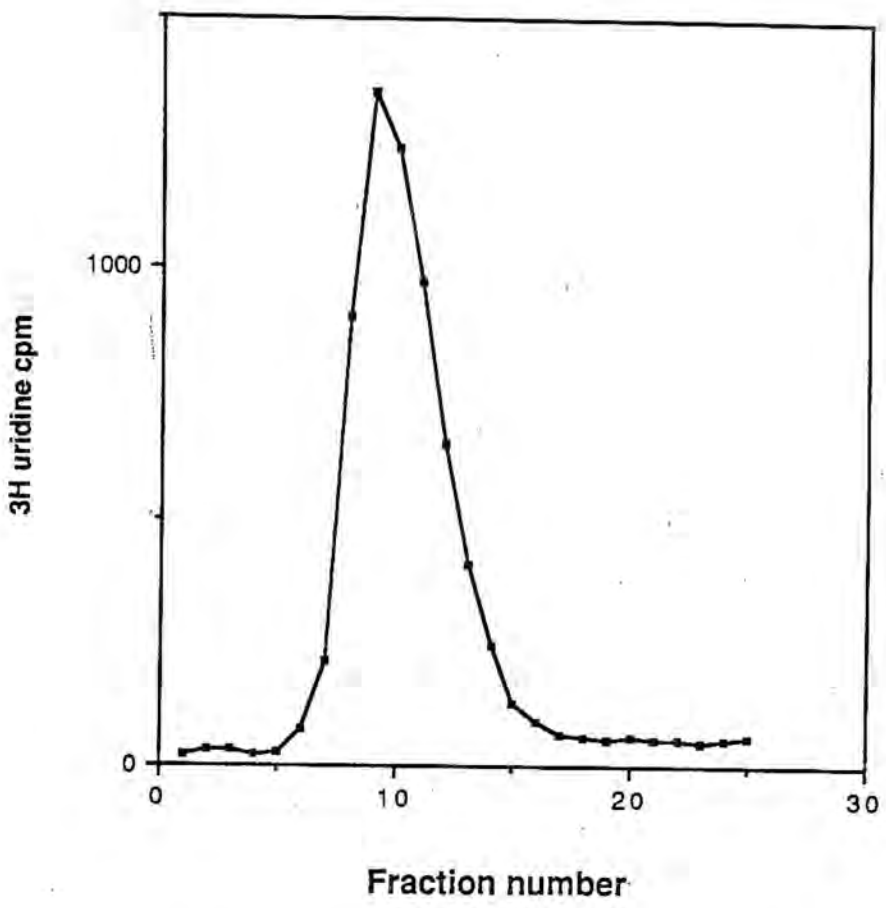


Figure 21. Sepharose CL-2B chromatography of purified EMCV replicative intermediate, obtained from AMT and UV light treated samples.

Fractions 8-11 (Figure 20) were pooled and ethanol precipitated and sujected to a second cycle of chromatography. Fractions 7-11 were pooled and used for electron microscopic studies as described under Methods.

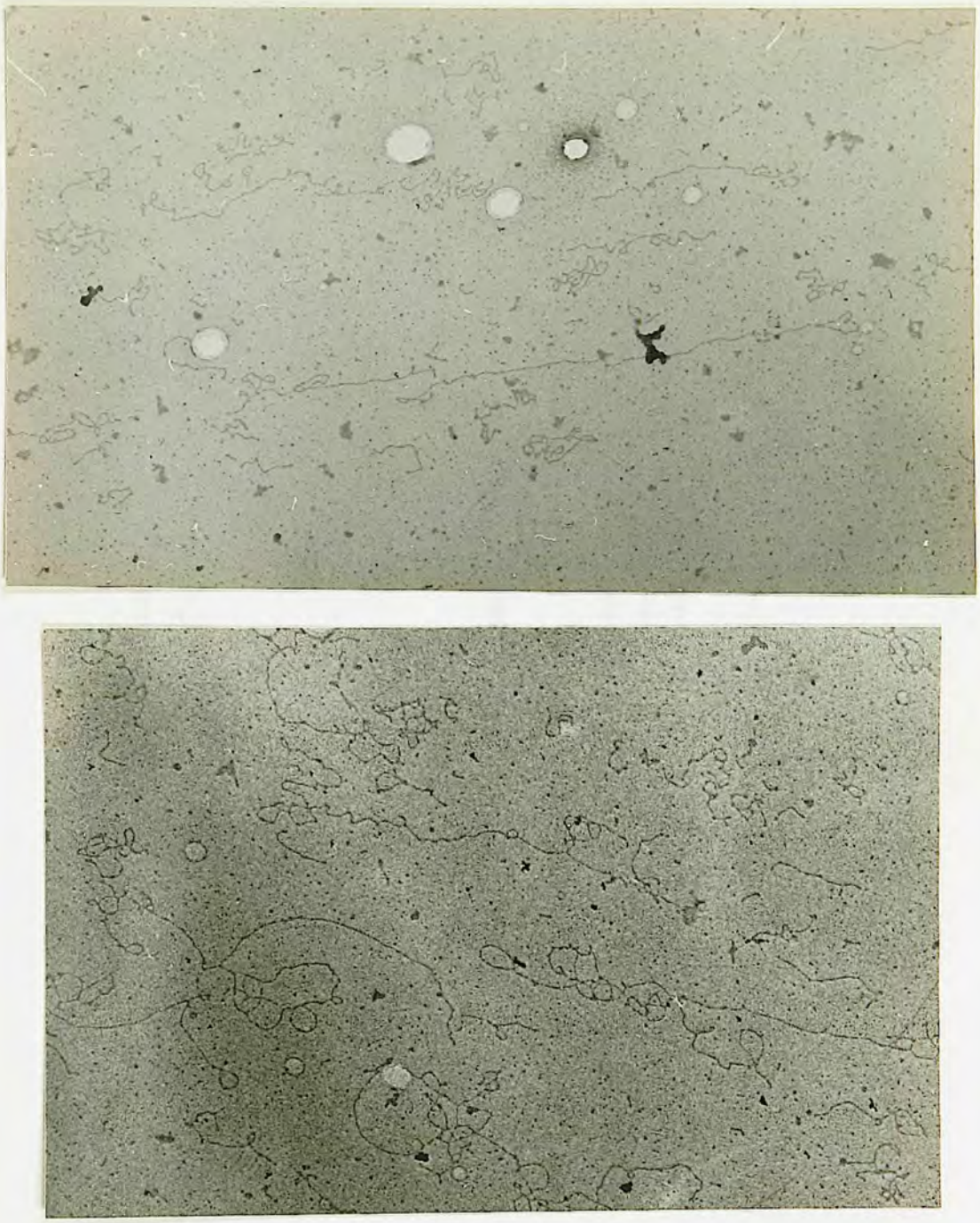


Figure 22. Electron micrographs of purified EMCV RI from cells treated with AMT in the absence of UV light.

Murine L cells were infected as described under methods. At four hours post-infection cells were treated with AMT and UV light. RI was purified and isolated as described under Methods, RNA was spread under denaturing conditions. Final magnification is 29,500x.

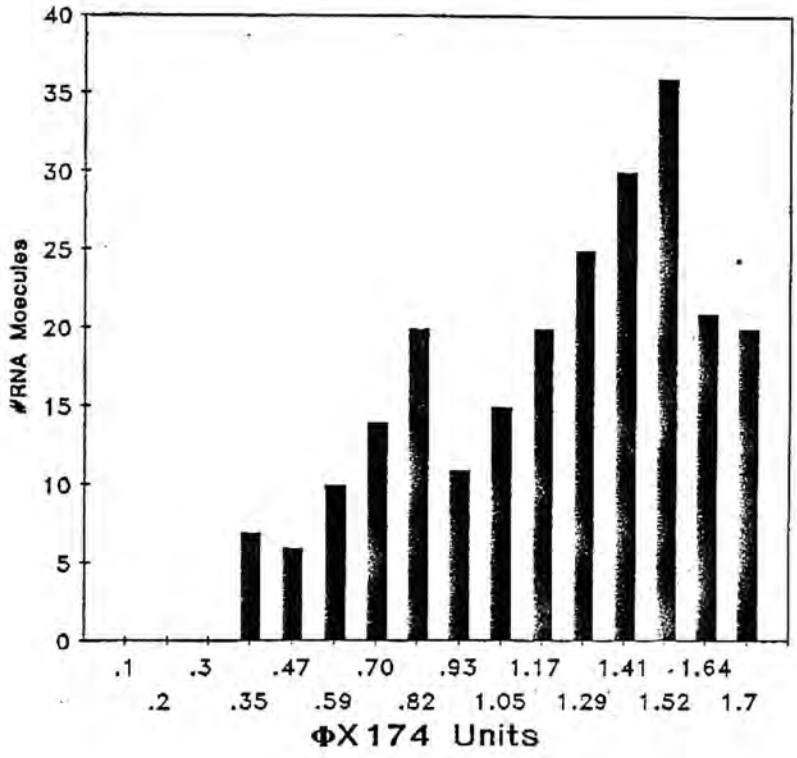


Figure 23. Histogram of RNA molecules obtained from intact cells treated with AMT, but without UV light.

Cells were infected as described under Methods. EMCV RI was purified and spread under denaturing conditions.

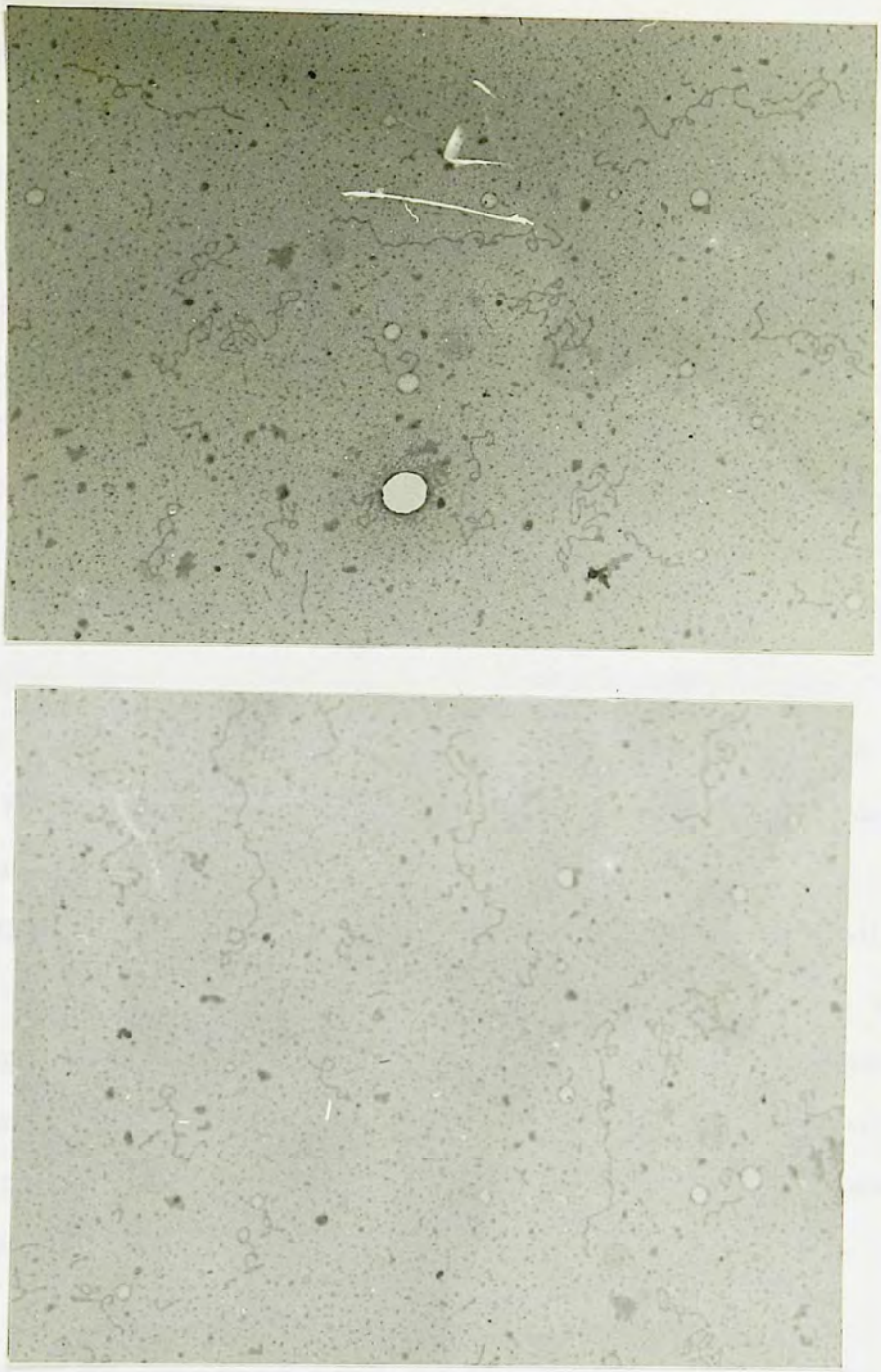


Figure 24. Electron micrographs of purified RI obtained from an <u>in-vitro</u> treatment of AMT.

Murine L cells were infected as described under Methods, S10 fraction was treated with AMT but not UV-irradiated. Purified RI was spread under denaturing conditions. Final magnification is 16,250x.

with RIs obtained from intact cells, RNA molecules treated in-vitro displayed a range of sizes, the mean size was determined to be 1.06 ± .36 ϕX174 units (Figure 25). The small decrease in the overall size of the RNA molecules may have resulted from RNase activity.

When spread under non-denaturing conditions (Figure 26A,B), the RI RNA isolated from EMCV-infected cells treated with AMT without UV-irradiation appeared as long and filamentous strands having 1-3 "bushes" (see arrows) attached to the backbone. The bushier RNA is probably collapsed ssRNA. The long backbone is presumed to be double-stranded regions of the RI. Measurement of these structures was not possible because in some cases the single-stranded branches of one molecule were complexed with another RI molecule. This may also explain why the RIs appear to be longer than those observed under denaturing conditions.

Electron microscopic analysis of RNA eluted from the included volume, and spread under denaturing conditions, showed a large degree of collapsed RNA which may represent cellular rRNA. RNA that appeared to be single stranded was also present. These molecules may be growing nascent chains from the RI's that separate from the parent strand upon denaturation (Figure 27).

V.I. EMCV RI isolated from cells treated with AMT and UV light

Psoralen modified RI from cells treated with AMT and UV light was spread under denaturing conditions as described under Methods. Unlike unmodified RI (Figure 22) these RNA molecules were significantly more compact, showed many loops and base-paired regions with few if any

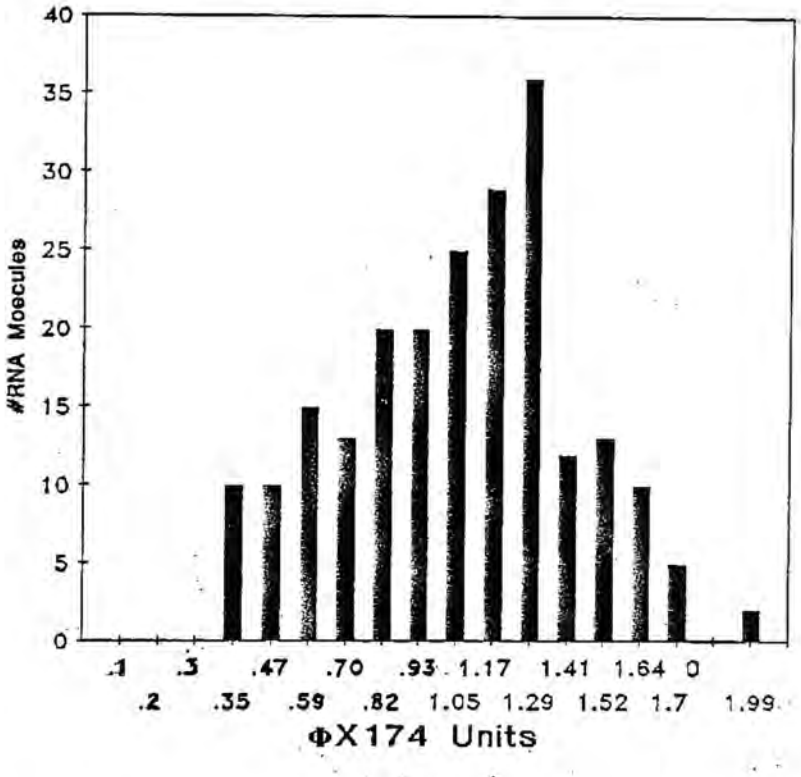


Figure 25. Histogram of RNA molecules obtained from <u>in-vitro</u> AMT treatment without UV light.

Murine L cells were infected as described under Methods. Purified EMCV RI was spread under denaturing conditions.

Figure 26. Electron micrographs of purified EMCV RI from cells treated with AMT without UV light spread under non-denaturing conditions.

RIs purified from intact cells infected with EMCV and treated without UV light, were spread under non-denaturing conditions as described under Methods. Final magnification is 20,475x.

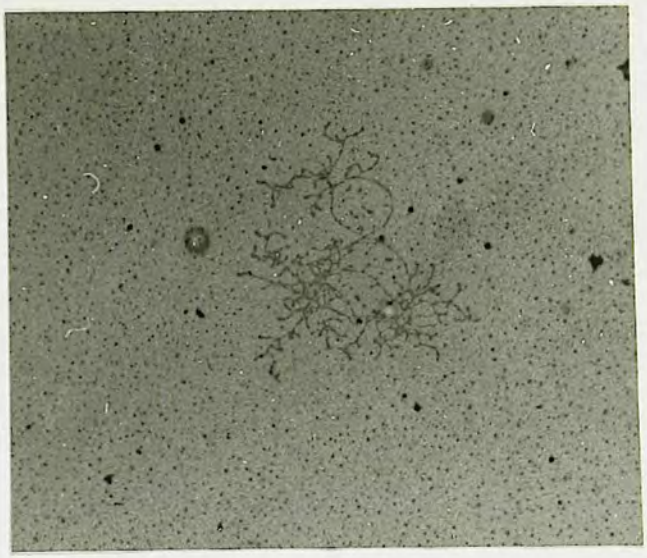
Figure 27. Electron micrograph of RNA in the included volume during purification of the RI.

Mouse L cells were infected with EMCV, and treated with AMT without UV light. RNA was purified as described under Methods. RNA in the included volume (Figure 16, fraction 13-25) was pooled and subjected to ethanol precipitation. RNA was spread under denaturing conditions. Final magnification was 33,500x.

observable single-stranded areas (Figure 28). These observations suggest that the structure of the RI is complex and contains significant secondary structure during replication. These RIs modified with psoralen in intact cells were similar to the structures observed upon spreading RI modified with AMT and UV light in-vitro (Figure 29 and 30). The major difference between these two samples was that the RI modified in intact cells (Figure 28) appeared to have more loops and hairpins compared to the RI modified in-vitro, which appeared less complex and with fewer branches (Figure 29 and 30). This difference may have resulted from differences in the concentration of RI's at the time of treatment. In the intact cell, RI's are probably fairly concentrated, as a result of being in a compact replicating complex. In contrast, the in-vitro treatment was carried out in a volume that was 3 times the packed cell volume. This may have diluted the replicative intermediates and/or altered their condensed structure, and/or decreased their ability to be modified with the psoralen.

RI from EMCV-infected cells treated with AMT and UV light but spread under non-denaturing conditions, (Figure 31A) appeared somewhat similar to those RI treated in-vitro (Figure 31B). As shown (see arrows), these RIs contain considerable single-stranded regions as exemplified by bush-like structures.

Electron microscopic analysis of RNA eluted from the included volume, spread under denaturing conditions, showed a large degree of collapsed RNA (containing secondary structure) which may represent cellular rRNA. Also present were denatured strands which may be short pieces of RNA from the RI (Figure 32).



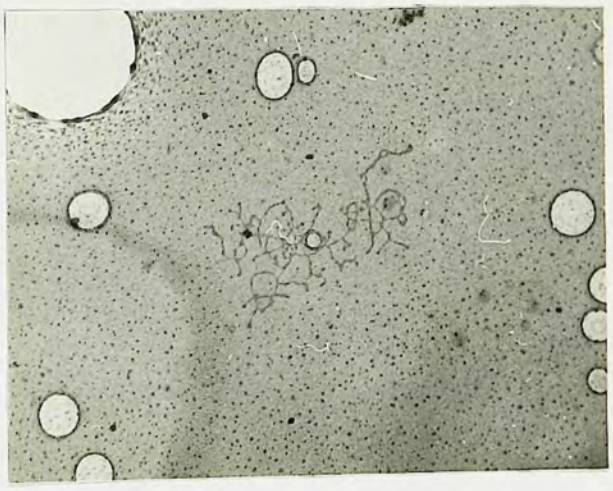


Figure 28. Electron micrographs purified EMCV-RI from cells treated with AMT and UV light, spread under denaturing conditions.

Purified RI obtained from intact cells treated with AMT and UV light were spread from a hypophase of 70% formamide. Final magnification of 29,500x.

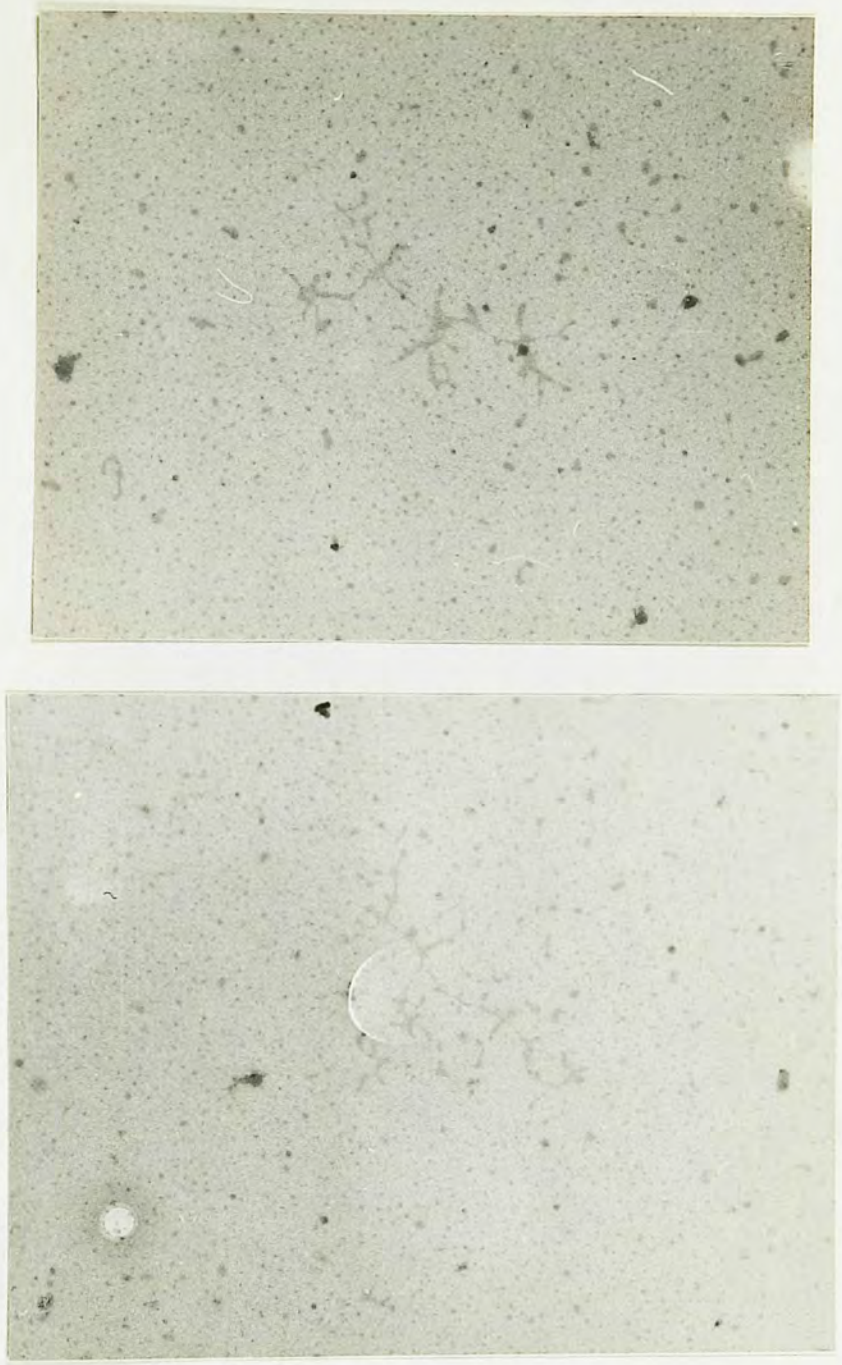


Figure 29. Electron micrographs of RI modified <u>in-vitro</u> with AMT and UV light.

Murine L cells were infected as descried under methods. At four hours-post infection S10 fractions were made and subsequently treated with AMT and UV light. RI was purified and isolated as described under Methods, RNA was spread under denaturing conditions. Final magnification is 37,000x.

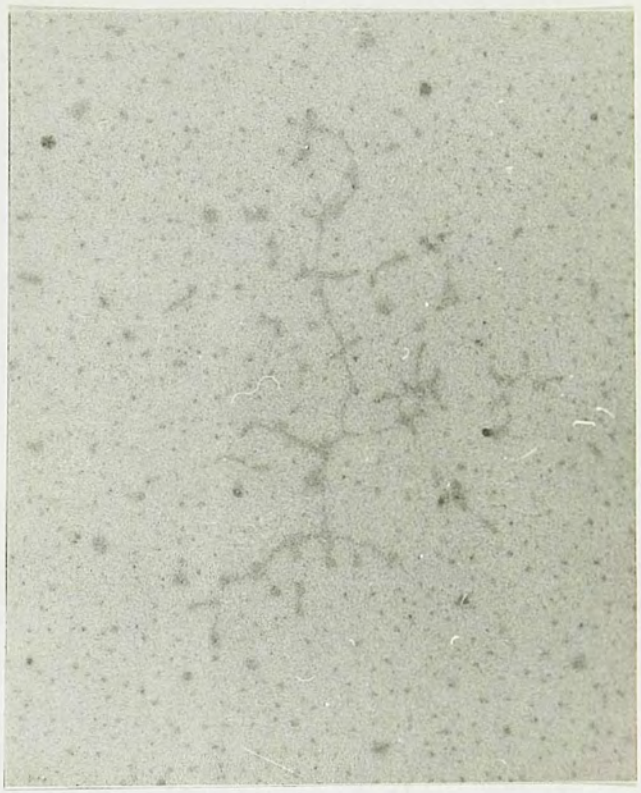


Figure 30. Electron micrograph of RI modified <u>in-vitro</u> with AMT and UV light.

Murine L cells were treated as described for Figure 29. RNA was spread under denaturing conditions. Final magnification is 48,000x.

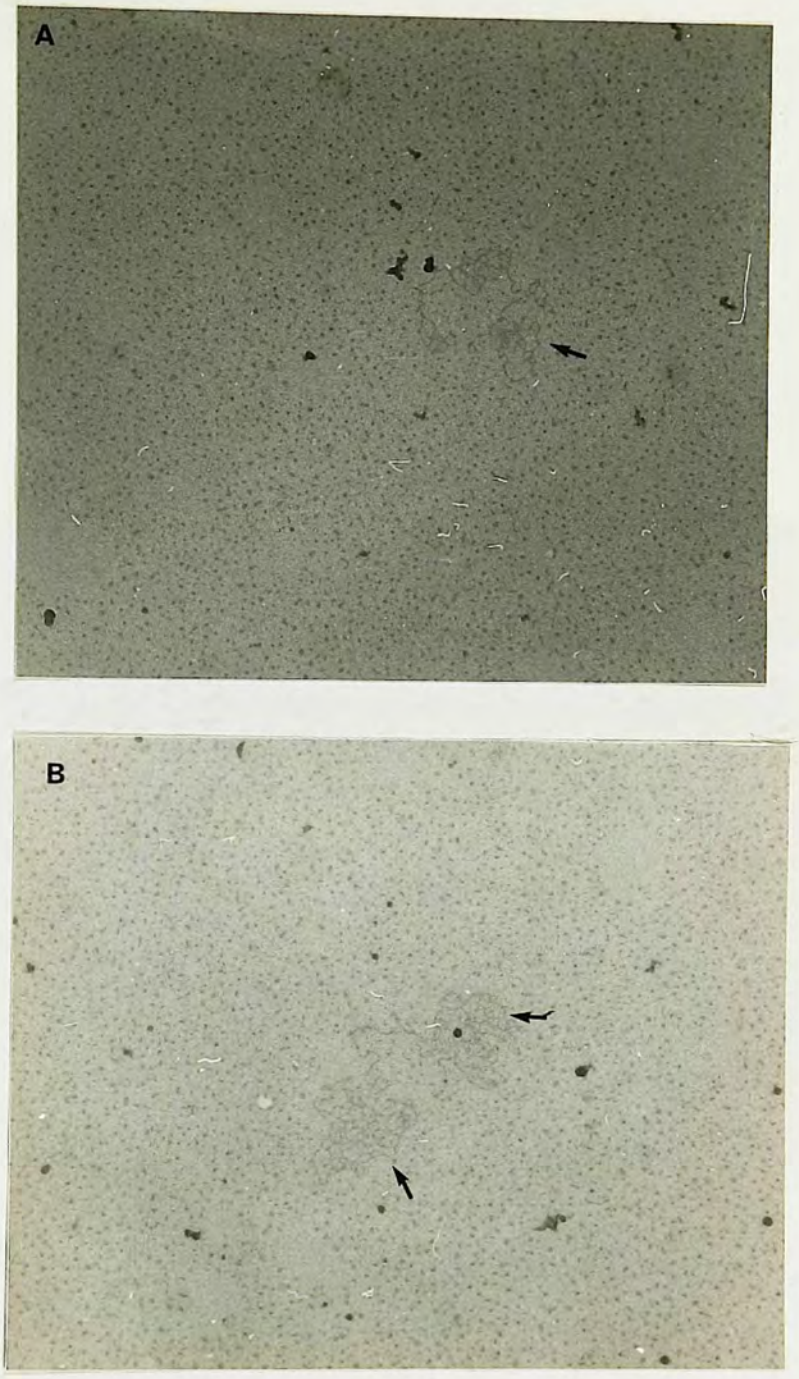


Figure 31. Electron micrographs of purified EMCV-RI treated with AMT and UV light spread under non-denaturing conditions.

Purified RI obtained from: (A) intact cells treated with AMT and UV light and (B) <u>in-vitro</u> treatment with AMT and UV light. RNA isolation as described under Methods, and spread from a hypophase of 30% formamide. Final magnification is 18,500x.

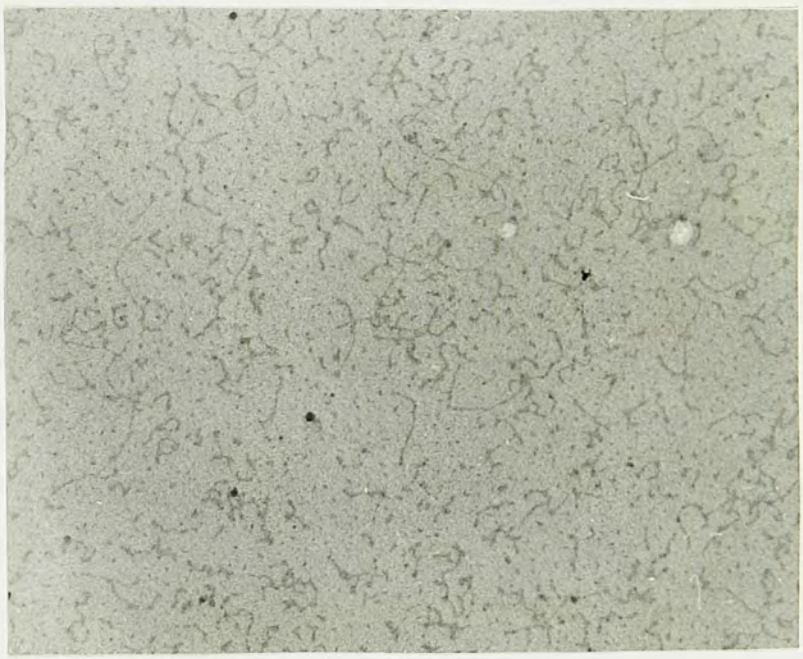


Figure 32. Electron micrograph of RNA in the included volume from EMCV-infected cells treated with AMT and UV light.

Murine L cells were infected with EMCV and treated with AMT and UV light. Fractions 13-25 (Figure 20) were pooled and subjected to ethanol precipitation. RNA was spread under denaturing conditions. Final magnification is 33,500x.

V.J. Electron micrographs of antibody-antigen complexes

Rabbit antibodies raised against psoralen-modified poly(A,U) were used to verify that replicative intermediates were modified by psoralen in intact cells. Characterization of the antibody demonstrated previously that antibody binds only to psoralen-modified RNA (Hurt et al., 1987).

Purified antibody was reacted with purified replicative intermediate from EMCV-infected mouse L cells treated with AMT and UV light. Antibody-RNA complexes were fixed and covalently cross-linked by treatment with formaldehyde and glutaraldehyde. Unbound antibody was separated from RNA-bound antibody by passage through a Sepharose 4B column. The peak eluting just after the void volume contained the RNA-antibody complex. Controls included reactions of preimmune rabbit IgG with AMT modified RI, and of immune IgG with unmodified RI (Figure 33). In both controls a major peak containing the majority if not all of the uncomplexed IgG eluted in the included volume. A small A280-containing peak eluted in the void volume representing uncomplexed RI. These results demonstrate an apparent absence of RNA-antibody interaction in the controls. In contrast, the appearance of a large A280-containing peak in the void volume of the sample containing immune antibody and psoralen-modified RI strongly suggests that AMT-RI interacts with this antibody.

Electron micrographs (Figure 34) revealed regions of the psoralen modified RI where the antibody reacted (see arrows). Electron micrographs of the void volume RNA from controls demonstrated that antibody did not react with control RNA treated with AMT alone (Figure 35A), and that the psoralen-modified RNA did not react with control

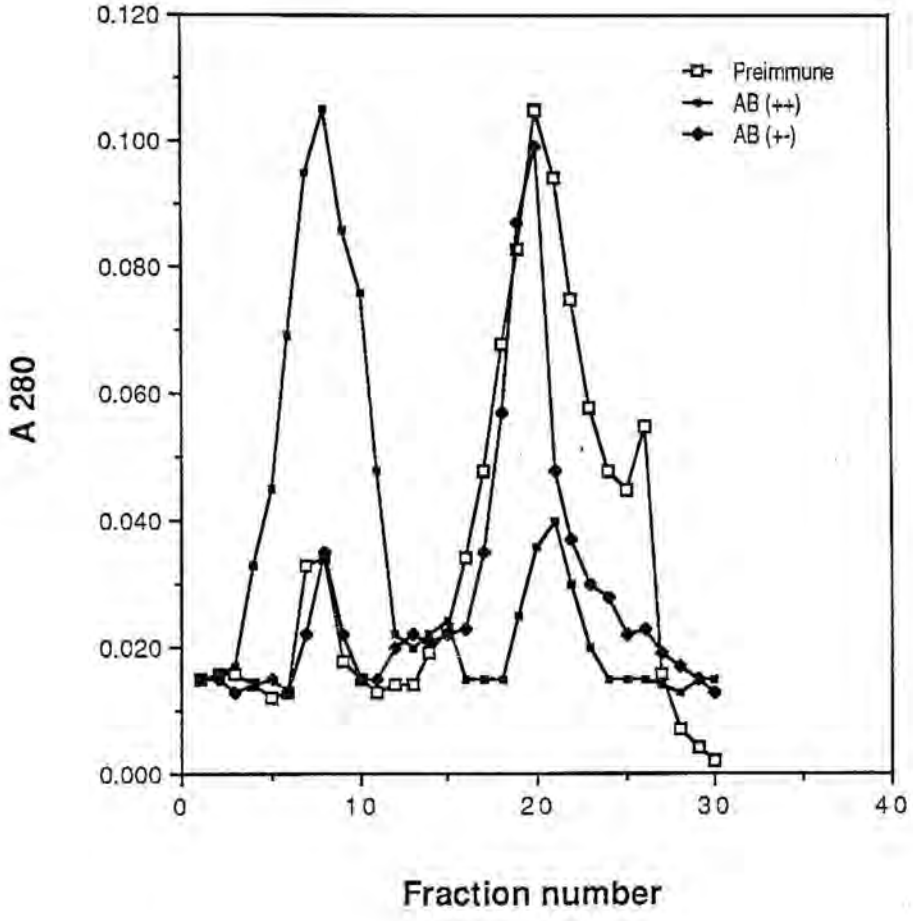


Figure 33. Sepharose 4B elution of RNA-antibody complex.

EMCV RI was isolated from intact cells treated with AMT and UV light (◆), or AMT without UV light (■), and complexed with 74b anti-AMT-poly(A,U) antibodies, or preimmune normal IgG (□). Complexes were fixed as described under Methods and chromatographed on Sepharose 4B.

Figure 34. Electron micrograph of purified EMCV RI treated with AMT and UV light complexed with purified antibody.

Murine L cells were infected with EMCV and treated with AMT and UV-irradiation. RI was purified as described under Methods and complexed with purified antibody specific to psoralen-modified nucleic acids. Final magnification 48,000x.

Figure 35. Electron micrographs of control RI-antibody complexes.

Murine L cells were infected with EMCV and treated with AMT with and without UV light. RI was purified as described under Methods. RI treated with AMT and UV light (A) was reacted with preimmune normal rabbit IgG. RI treated with AMT without UV light (B) was reacted with immune antibody. Final magnification 48,000x.

preimmune IgG (Figure 35B). These results demonstrate that under the conditions used, the EMCV replicative intermediates are modified by the psoralen derivative AMT, and therefore probably contain base-paired regions in intact cells.

V.K. Electron micrographs of RI obtained from interferon-treated L cells

RI obtained from interferon-pretreated L cells, treated with AMT and UV light after infection, was spread under denaturing conditions (Figure 36A). The RNA was significantly shorter than EMCV RI from cells not treated with interferon (compare Figure 36A with Figure 28). Similar results were obtained in the absence of UV-irradiation (compare Figure 36B with Figure 22). The size distribution in the interferon-treated cells ranged from .19 to .34 X174 units (Figure 37A and B) compared to .35 to 1.64 X174 units in control cells (Figures 23 and 25).

These results support the model based on <u>in-vitro</u> experiments proposed by Nilsen & Baglioni (1979). These workers reported that radiolabeled ssRNA containing a poly(A) tail bound to a poly(U) segment is preferably degraded by RNase L in extracts from interferon-treated cells. The current results provide evidence which strongly suggests that EMCV RNA is degraded in interferon-treated cells. This degradation may result from activation of 2-5A synthetase by double-stranded regions present in the replicative intermediate, and subsequent activation of RNase L by 2-5A.

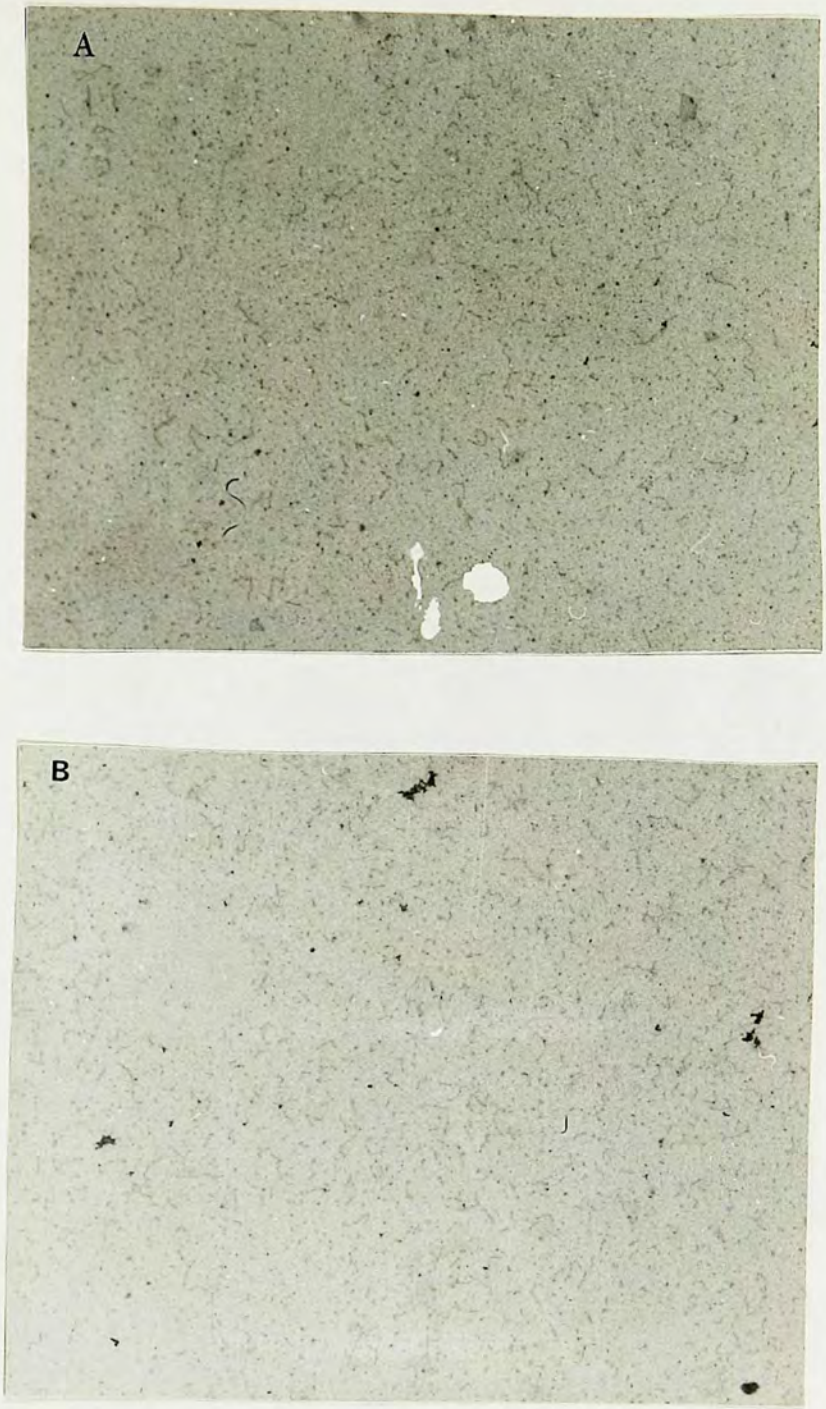


Figure 36. Electron micrograph of RI from interferon-treated murine cells.

Murine L cells were pretreated with interferon (500 U/ml) and infected with EMCV. Cells were (A) treated with AMT and UV light or (B) treated with AMT without UV light. Purified RNA was spread under denaturing conditions. Final magnification 28,800x.

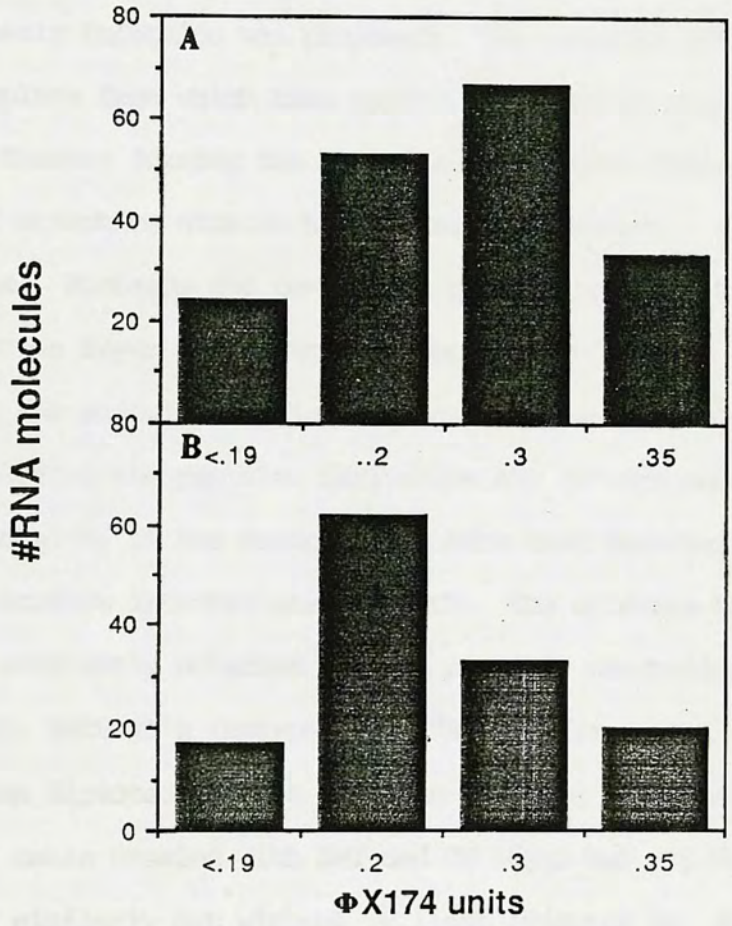


Figure 37. Histogram of RNA molecules obtained from L cells pre-treated with interferon.

Cells were treated with interferon (500 U/ml) for 18 hours prior EMCV infection. Following infection, cells were treated with (A) AMT without UV light, (B) AMT and UV light.

Conclusions

A mechanism for picornavirus replication has been proposed previously based on analysis of replicative intermediates using various biochemical techniques such as sedimentation, RNase resistance and electron microscopic work (Lundquist & Maizel, 1978, Meyer et al., 1978, Bishop & Koch, 1969, Richards et al., 1984). From these studies a model for early infection was proposed: the parental strand (+) is used as a template from which base paired (-) daughter strands are synthesized, thereby forming the branched replicative intermediate. The number of branching strands has varied in different investigations. Richards and co-workers report from none to twelve branches, whereas Meyer and co-workers report from none to six branches with the majority bearing 1-2 branches per RNA molecule.

By employing the psoralen derivative AMT (4'-aminomethyl-4,5', 8-trimethylpsoralen) it was demonstrated here that base-paired regions occur in replicative intermediates of EMCV. The evidence that psoralen was covalently attached to, and possibly cross-linked, with the RNA was (i) EMCV RI's incorporated [^{3}H]-AMT (Figures 14 and 15), (ii) antibodies directed against psoralen-modified RNA bound RI from EMCV-infected cells treated with AMT and UV light but not RI from cells treated similarly but without UV light (Figures 33, 34, and 35), and (iii) electron microscopic analysis of replicative intermediates from cells treated with AMT and UV light showed a distinctive and complex molecule that contained significant secondary structural characteristics (Figure 28). These data suggest that during EMCV replication at least some base-pairing occurs.

The results described here are in agreement with results obtained by Nilsen et al., (1981). These workers reported a dose dependent inhibition of EMC viral RNA synthesis by AMT, and correlated this effect with resistance of the RNA to RNAse digestion after heating to 105°C and quick cooling. Specifically, RI from cells treated with AMT and UV light were more resistant to RNase than RI from control cells. The authors concluded that the nascent strands in controls were released by the treatment, whereas cross-linked strands snapped-back to the template and yielded a double-stranded "core" that was resistant to RNase A. These indirect results suggest that AMT intercalated and modified the RI by forming cross-links or monoadducts that subsequently rendered the RI resistant to RNase A.

In contrast, polio RI from cells treated with AMT and UV light show an architecture that was very different from that reported here. Richards and co-workers (1984) reportedly cross-linked polio RI in-vitro and compared its structure under non-denaturing conditions to the structure of polio RI isolated from infected cells. They concluded that AMT does not alter the structure of polio RI and that extensive base-pairing does not exist in intact cells. The results here, in contrast, suggest that AMT does alter the structure of EMCV RI and that base pairing does occur in intact cells. This may result if polio RI has less extensive base-pairing than EMCV RI. One possible mechanism to explain this discrepancy is that polio virus may initiate replication more efficiently than EMCV. It has been observed that polio RI has on the average 4 nascent strands per template, whereas EMCV RI has 1 or 2 nascent strands (Figure 26), (Richards et al., 1984, Thach et al., 1974). The presence of fewer polymerase

molecules within EMCV RI may allow for more extensive base-pairing during replication.

It has been proposed that during viral infection, or any cellular condition in which the 2',5'-oligoadenylate system is induced, the presence of double-stranded RNA activates 2-5A synthetase and leads to elevated levels of 2-5A. 2-5A then activates a latent endoribonuclease that degrades viral RNA. Williams et al., (1979) were the first to demonstrate production of 2-5A in EMCV-infected cells pre-treated with interferon. Nilsen & Baglioni (1979) reported that ssRNAs containing a region of dsRNA is prefentially degraded in comparison to ssRNA that contains no significant regions of dsRNA. These experimentts were carried out in-vitro using extracts of interferon-treated cells that contained elevated levels of 2-5A synthetase. However, it has not been demonstrated that viral RNA that contains dsRNA regions is preferentially degraded in intact cells. The electron microscopic studies here demonstrate that RI from interferon-treated cells was significantly smaller compared to RI from control cells. Although this does not demonstrate preferential degradation of viral vs cellular RNA, it is the first direct evidence of probable interferon-mediated degradation of EMCV RNA in intact cells, and supports the postulate that the 2-5A system plays a role in the interferon-mediated inhibition of EMCV replication.

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